# IMMUNOLOGY OF BCG VACCINATION IN MICE: IMPLICATIONS FOR TUBERCULOSIS VACCINATION AND FOR THE USE OF BCG AS A RECOMBINANT VACCINE VECTOR

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| **TITLE OF THESIS** | Immunology of BCG Vaccination in Mice:<br>Implications for Tuberculosis Vaccination and<br>For the use of BCG as a Recombinant Vaccine Vector |
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## ABSTRACT

The effect of vaccination dose on the immune response of mice to *Mycobacterium bovis* Bacille Calmette Guerin (BCG) was examined. To assess T helper cell-type 1 (Th1) and -type 2 (Th2) responses, a very sensitive ELISPOT assay was developed. Using this assay, the numbers of BCG-specific interferon γ- and interleukin 4-producing spleen cells were used as indicators of the Th1 and Th2 responses, respectively. IgG1 and IgG2a serum antibody titers were also assessed. Mice were immunized with different doses of BCG, and their immune response to BCG antigen was determined at various times after vaccination. Low dose immunized mice developed predominantly Th1 responses and little antibody, while those given higher numbers of bacilli generated a mixed Th1/Th2 response and had significant antibody titers. Furthermore, mice given the lower doses of BCG developed an immune imprint that enabled them to resist development of a Th2 response on subsequent challenge with a higher dose of BCG, which generates a Th2 response in previously unexposed mice. The immune response of mice immunized with recombinant BCG (rBCG) expressing *Escherichia coli* β-galactosidase was also characterized. Th1 cells predominated in the immune response to BCG antigen as well as to the expressed β-galactosidase antigen in low dose vaccinated mice. Moreover, the Th1 response to both antigens was stable after high dose challenge of low dose vaccinated animals indicating that immune imprinting had been established both to BCG and to the recombinant β-galactosidase antigen.

The results indicate that the immune state that develops in mice upon vaccination with BCG depends on the initial dose of antigen administered, with low amounts of antigen leading to Th1 immunity, and relatively high amounts of antigen leading to Th2 immune responses. *Mycobacterium tuberculosis* is only controlled effectively by a cell-mediated immune response. Immunization with a relatively low dose of BCG, rather than the current standard dose, may provide greater immune protection against *M. tuberculosis*. Moreover, low dose vaccination with rBCG expressing proteins of leishmania or HIV promises to be an effective means of vaccination against diseases caused by these pathogens, which can only be contained by a Th1 response.

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| | |
|---|---|
| AFB | Acid-Fast Bacilli |
| APC | Antigen-Presenting Cell |
| BCA | Bicinchoninic Acid |
| BCG | Bacille Calmette Guerin |
| β-gal | Beta Galactosidase |
| BSA | Bovine Serum Albumin |
| cfu | Colony-Forming Units |
| CMI | Cell-Mediated Immunity |
| CTL | Cytotoxic T Lymphocyte |
| DNA | Deoxyribonucleic Acid |
| DTH | Delayed-Type Hypersensitivity |
| EIA | Enzyme Immunoassay |
| ELISPOT | Enzyme-Linked Immunospot Assay |
| eTh | Effector T Helper Cell |
| HA | Influenza Hemagglutinin |
| HIV | Human Immunodeficiency Virus |
| HLA | Human Lymphocyte Antigen |
| hsp60 | Heat Shock Protein 60 |
| i.d. | Intradermal |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| IFNγ | Inteferon Gamma |
| IgG1 | Immunoglobulin-γ-1 |
| IgG2 | Immunoglobulin-γ-2 |
| IgG2a | Immunoglobulin-γ-2a |
| IgA | Immunoglobulin-α |
| IL-2 | Interleukin 2 |
| IL-4 | Interleukin 4 |
| IL-5 | Interleukin 5 |
| IL-6 | Interleukin 6 |
| IL-10 | Interleukin 10 |
| IL-12 | Interleukin 12 |
| IL-13 | Interleukin 13 |
| IL-18 | Interleukin 18 |
| IRF-1 | Interferon Gamma Regulatory Factor 1 |
| $K^r$ | Kanamycin Resistance |
| LPS | Lipopolysaccharide |
| MHC | Major Histocompatibility Complex |
| mRNA | Messenger Ribonucleic Acid |
| NA | Influenza Neuraminidase |
| NP | Influenza Nucleoprotein |
| Nramp | Natural Resistance-Associated Macrophage Protein |
| ORF | Open Reading Frame |
| OspA | *Borrelia burgdorferi* Outer Surface Protein A |
| PBMC | Peripheral Blood Mononuclear Cells |
| PBS | Phosphate Buffered Saline |

| | |
|---|---|
| pi | Post Infection |
| PPD | Purified Protein Derivative |
| pspA | *Streptococcus pnemoniae* Surface Protein A |
| pTh | Precursor T Helper Cell |
| rBCG | Recombinant BCG |
| rBCGβgal | Recombinant BCG Expressing β-Galactosidase |
| $rdH_2O$ | Reverse Osmosed Distilled $H_2O$ |
| RI | Reactive Intermediate |
| RNA | Ribonucleic Acid |
| RR | Reactive |
| s.c. | Subcutaneous |
| SD | Standard Deviation |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SIV | Simian Immunodeficiency Virus |
| TB | Tuberculosis |
| TBS | Tris-buffered saline |
| TCR | T Cell Receptor |
| TGF-β | Transforming Growth Factor-β |
| Th | T Helper Cell |
| Th1 | T Helper Cell Type 1 |
| Th2 | T Helper Cell Type 2 |
| TNF | Tumor Necrosis Factor |
| tRNA | Transfer Ribonucleic Acid |
| $T_SAb$ | Suppressor T Cells for Antibody |
| $T_SDTH$ | Suppressor T Cells for DTH |
| TTBS | Tris-buffered Saline with Tween 20 |
| UI | Unreactive Intermediate |
| UU | Unreactive |
| WHO | World Health Organization |
| XRBC | Xenogenic Red Blood Cell |

# 1 INTRODUCTION

## 1.1 Low Dose Antigen Vaccination

In 1958, Salvin demonstrated that guinea pigs immunized with ovalbumin or diptheria toxoid in such a way that they developed an Arthus-type reaction, went through a period in which they displayed a delayed type hypersensitivity (DTH) response to the antigen (Salvin, 1958). After an initial latent period, the animals developed DTH that was replaced several days later by Arthus-type hypersensitivity. The time required to convert from the DTH to Arthus response was inversely proportional to the sensitizing dose of antigen. Furthermore, when very low sensitizing doses of antigen were administered, only DTH responses developed (Salvin, 1958). The results suggested that the immune response follows a particular pattern of development. The initial, and often the only response to develop is of the cell-mediated type, followed thereafter by a humoral response if the immunizing conditions are appropriate. I believe this to be a general principle of the immune system, that cell-mediated immune responses will always precede antibody responses in the developing immune response. Recent studies by Lederer et al. (1996) and Bird (1998) suggest that indeed interferon γ (IFNγ) preceeds interleukin 4 (IL-4) in the developing immune response. The studies of Salvin (1958) and Parish (1972) demonstrated that the dose of antigen administered has a significant impact on the type of immunity that is induced, with low doses favouring DTH and high doses favouring progression to an antibody response.

Asherson and Stone (1962) demonstrated that the development of an antibody response inhibited the ability of mice to develop DTH responses to the same antigen, a state referred to as humoral immune deviation. The work of Parish suggested that the

inverse was also true. Rats immunized with low doses of antigen administered repeatedly over a number of days developed an exclusive and stable DTH response, and were unable to develop a significant antibody response (Parish, 1972). These observations suggested that reciprocal control exists over the two arms of the immune response. Immunization in a manner that induces a stable cell-mediated immune response could prevent the subsequent development of an antibody response and vice versa. Cells capable of suppressing DTH ($T_sDTH$) and antibody ($T_sAb$) responses were identified (in modern terminology) as $CD4^+$ and $CD8^+$ cells, respectively (Ramshaw et al., 1976; 1977).

T helper (Th) cells are the choreographers of the immune response, involved in directing and controlling almost all aspects of immunity. In a simplified classification system, they are divided into two distinct groups based on the cytokines they produce (Mosmann and Coffmann, 1987). Type 1 T helper cells (Th1 cells) mediate DTH and help to activate cytotoxic T cells. They are characterized by production of IFNγ, interleukin 2 (IL-2), lymphotoxin and tumor necrosis factor (TNF) (Abbas et al., 1996; O'Garra, 1998). Type 2 T helper cells (Th2 cells) produce IL-4 and also interleukin 5 (IL-5), interleukin 6 (IL-6) and interleukin 13 (IL-13) (Abbas et al., 1996; O'Garra, 1998) and are important for antibody production and immunity to parasitic infections. No doubt there are many subgroups within this classification, with cells producing various combinations of these cytokines. For the purpose of this discussion, cells that produce IFNγ are classed as Th1 cells, whereas those that produce IL-4 are termed Th2 cells. This terminology will serve as IL-4 and IFNγ represent cytokines that are dominant in regulating responses at opposite ends of the immune spectrum.

Some infectious agents can be controlled by an antibody (Th2) response while others are only effectively eliminated by a cell-mediated (Th1) immune response. The induction of an inappropriate response will be ineffective at preventing or curing disease and may even be exacerbating (Sher et al., 1992). It is therefore imperative that the

appropriate immune response be generated with vaccination. Leishmania parasites are an example of a disease for which a cell-mediated response is required to provide effective immunity ( Locksley and Scott, 1991; Locksley et al., 1999). Inbred mouse strains can be classified as susceptible or resistant to leishmania infection depending on the type of T helper cells that they generate upon infection with a relatively large number of parasites. Those strains that develop a Th1 response resist the infection and cure the lesion, while the infection progresses in those that mount a Th2 response. In an interesting set of experiments, Bretscher et al. demonstrated that BALB/c mice, the prototypic susceptible strain, were able to resist an infection with a low number of parasites and, in time, became immune to a larger, normally pathogenic dose. Analysis of the immune response showed that the low dose infection induced a Th1 response, which was maintained even after the high dose exposure (Bretscher, 1992a; Menon and Bretscher, 1996; 1998). This work not only demonstrated the efficacy of low dose infection for inducing Th1 cells, but also demonstrated that immune deviation or imprinting can be achieved by this method. This is an important aspect for vaccine research as it demonstrates that a polarized response can be achieved and also maintained upon subsequent challenge.

Mycobacteria are similar to leishmania in that they are both slow growing intracellular organisms that infect cells of the monocyte lineage, and a Th1 response is necessary to provide protection from both pathogens. In this thesis, I have characterized the immune response of mice after vaccination with Bacille Calmette Guerin (BCG), an attenuated strain of *Mycobacterium bovis*, and recombinant BCG (rBCG). The implications of the observations are discussed in the context of immune regulation and vaccination against tuberculosis (TB) and other diseases.

## 1.2 Tuberculosis

### 1.2.1 Mycobacteria

The genus *Mycobacterium* represents a group of over seventy organisms that are grouped together based upon acid alcohol fastness, properties of mycolic acids in their cell wall and DNA guanine and cytosine content of 61-71 mol % (Shinnick and Good, 1994). The genus is further divided into two groups based on growth rate; slow growers require more than seven days to form visible colonies on solid medium and fast growers that form colonies in less than seven days. In humans and other animals, the slow growing group is responsible for most mycobacterial disease, whereas the fast growing species are generally non-pathogenic. Members of the *Mycobacterium tuberculosis* complex are the etiologic agents of TB (reviewed in Shinnick and Good, 1994). The complex includes three species: *M. tuberculosis, M. bovis* and *M. africanum*, which share >95% DNA homology with each other. The *M. avium* complex (*M. avium* and *M. intracellulare*) also causes pulmonary and non-pulmonary infections, primarily in immunocompromised individuals.

### 1.2.2 Epidemiology

Tuberculosis has been afflicting humans since before the beginning of recorded history. Evidence of the disease has been identified in skeletal remains as old as 8000 years (Stead and Dutt, 1994) and the disease continues to be the most prevalent infectious cause of human morbidity and mortality in the world today. Developing countries where proper medical care and access to antibiotics is limited currently account for the majority of cases. The World Health Organization (WHO) estimates that 8 million new cases of TB arise each year and 3 million people die annually due to the disease (World Health Organization, 1994).

When a disease epidemic first afflicts a population, morbidity and mortality rates follow a typical curve over time. This curve rises relatively rapidly, reaching a peak

followed by a much slower and longer descent, as the pool of susceptible individuals in the population is reduced either through acquisition of immunity or through death. Tuberculosis, like other contagious diseases, follows this typical pattern when introduced into a particular environment. In TB epidemics, this pattern of disease is explained by natural selection and the elimination of susceptible individuals from the population (Grigg, 1958; Stead and Dutt, 1994). However, TB is a slow disease, with patients often living many years between the time of infection and death. Because of this, the epidemic wave of TB is much longer than for most diseases. The time for a TB epidemic to run its course is estimated at 300 years or more (Grigg, 1958). In most western countries, the current TB epidemic is in the late stages and TB rates have fallen significantly over the last century. As the infection moves through a community, those who are more susceptible to the disease are affected and eventually killed by the disease, while the resistant individuals remain alive to pass on their resistance genes to their children. As the pool of susceptible individuals is depleted, fewer people become ill following infection and eventually the epidemic becomes self-limiting. In some countries in Africa and Asia, the epidemic is in an early stage (Cummins, 1920), and morbidity and mortality rates are currently higher. In these communities, the current epidemic wave started at a much later time. Dramatic evidence of the early part of the TB wave was observed in North American Indians after they had settled on reserves in the 1880s. Ferguson (1955) reported that the death rate from TB rose from approximately 1000 deaths per 100,000 people in 1881 to 9000 per 100,000 in 1886, almost a ten-fold increase in a span of five years. This was at the peak of the TB epidemic in this group. The severe annual death rate was most probably a result of a population that had little previous exposure to TB being placed in conditions ideal for TB spread. TB rates among the native people of Saskatchewan have continued to fall over the past century, but remain higher than among the general population (Hoeppner, 1991; Hoeppner and Marciniuk, 2000). Other examples of high TB mortality as a result of introduction of the pathogen into a population come from the reports of

Cummins (1920) that have compared TB rates in British troops to those of troops recruited into British military service from other countries.

TB patients can be divided into two groups: those with progressive primary disease (early progressive) and those with reactivation disease (recrudescent) (Moulding, 1994; Stead and Dutt, 1994). Individuals with progressive primary disease are infected with *M. tuberculosis* and develop disease as a result of the primary infection. Those with reactivation disease initially control the infection, but eventually develop disease, usually years after the initial exposure, as a result of a failure of the immune system to continue to control that infection. With regards to the epidemic wave, primary infection is more interesting, as disease is a direct result of susceptibility to infection, rather than the result of a later event that subsequently undermines an initial protective response. In endemic areas, the type of disease seen in childhood TB is quite different from that seen in adults, being more generalized and progressive. Interestingly, TB in those who are exposed for the first time as adults is often similar to the childhood form (Cummins, 1920).

In the early stages of a TB epidemic, disease occurs more often in children as susceptible individuals develop disease as a direct result of primary infection (Grigg, 1958). In the later stages of the epidemic wave, as natural selection works to eliminate those susceptible to progressive primary disease, the majority of cases of TB occur in adults, often as a result of reactivation. Reactivation disease occurs in individuals whose infection occurred at a time when TB was more prevalent, often years previously, (Stead and Dutt, 1994) and hence it contributes to the prominence of adult TB at the end of the epidemic wave. This etiology is common in non-endemic areas. Alternatively, in endemic areas, adults may develop disease as a consequence of re-infection or reactivation.

The pattern of a TB epidemic has particular significance for the situation here in Canada. In this country, the TB rate is similar to that in other developed countries among the general population. However, among the indigenous people, the rate of TB cases is considerably higher than in Caucasians (Hoeppner, 1991; 1998). One possible

explanation for this is that the native people are at an earlier stage in the epidemic wave than Caucasians, having only begun the recent epidemic wave in the last 150 years (Ferguson, 1955; Hoeppner and Marciniuk, 2000). If more individuals in these Native communities are susceptible to progressive primary infections, a higher rate of TB is expected in children among the Native population than in individuals of European descent. This is certainly the trend in Saskatchewan as the majority of TB cases in Native People occur in children under five years of age while in the Caucasian population most cases occur in the elderly (Hoeppner, 2000).

### 1.2.3 Tuberculosis Pathology

Since the tubercle bacillus was identified in 1882 (Koch, 1932), scientists have attempted to understand the relationship of the organism with the infected host. After more than one hundred years of research, there are still many questions to be answered, and the disease remains somewhat of an enigma. Understanding the disease pathology and progress in an individual requires an understanding of the immune response generated in that individual to *M. tuberculosis* and perhaps to other mycobacterial species.

The majority of individuals who are infected with *M. tuberculosis* do not become ill with TB. In fact, 90-95% of individuals who have been infected with *M. tuberculosis*, as assessed by a positive skin test, do not develop disease (Sheffield, 1994). In these individuals, the immune system is able to respond to the organism, generating an immune response which is capable of controlling the growth and spread of the organism, and preventing clinical disease from developing. In the remaining individuals, the immune system somehow fails to generate either sufficient or appropriate immunity to control the organism and these individuals develop some form of clinical disease.

Understanding the pathology in *M. tuberculosis* infections is further complicated by the many forms of disease which develop: primary vs. secondary, multibacilliary vs. paucibacilliary, pulmonary vs. extrapulmonary, and the many variations and gradations which are seen within each group. To better understand the immune response to *M. tuberculosis* and its role in development of disease, it will be necessary to understand the distinctions between these groups. Most importantly, it is essential to distinguish between primary progressive disease and secondary or reactivation disease, as these different forms are likely the result of very different mechanisms. In the former case, the disease results as an initial failure of the immune system to respond appropriately to the *M. tuberculosis*. In the latter, the disease occurs as a subsequent failure of the immune system to continue to contain the primary infection, often as a result of immune suppression of some kind. The reason for the immune system's failure to continue to control the infection is known in many of these cases, whether it is from circumstances associated with immunosuppressive diseases such as human immunodeficiency virus (HIV), malnutrition, alcoholism or concomitant infection ( Stead, 1967; Sheffield, 1994). Therefore, the discussion presented here will focus mainly on why patients fail to contain a *M. tuberculosis* infection upon primary exposure. I feel that this is a much more interesting question from the immunological standpoint and that answering this question will lead to a far greater understanding of the pathogen/host relationship and of the disease itself.

Tuberculosis can affect virtually any body tissue, but initial infection in humans is almost invariably by the respiratory route, from which it subsequently spreads to other areas (Stead, 1967; Dannenberg, 1994). In order to be infective, aerosol particles containing mycobacteria must be very small to ensure that they remain suspended in air long enough to reach the alveoli of the lung where the infection takes place (Ferguson, 1955; Dannenberg, 1994). It has been estimated that aerosols of one to three bacteria will stay suspended long enough to reach the alveoli while larger particles will generally settle

on mucosal surfaces before reaching the alveoli and be cleared by cilia (Dannenberg, 1994). Bacilli that lodge in the alveoli will be taken up by alveolar macrophages that are resident in the lung. Many of the bacilli will be dead upon inhalation or will be killed by the action of non-specifically activated macrophages. If the macrophage does not possess the capacity to kill the engulfed tubercle bacillus, then the bacteria can grow intracellularly, and the initial lesion may be established (Dannenberg, 1994). Evidence suggests that in the majority of cases, a TB infection is the result of a single infectious particle as most infections start at a single focus (Ferguson, 1955). If this is true, then the degree of exposure will increase the likelihood of infection, but not the eventual outcome. In other words, individuals who live in endemic areas are more likely to become infected, but the probability of developing disease once infected, or the severity of disease, depends on other factors. Epidemiological evidence supports this theory as the rate, type and severity of TB in recently converted skin test-positive individuals who are at a high risk of infection does not differ significantly from similar individuals who are at a low risk of infection (Ferguson, 1955; Hoeppner, 2000).

I propose that the development of tuberculous disease in any individual is the result of an inability of the immune system to control the infection. The reasons for failure of the immune system will not be the same in all patients. Defining the reason for such failure in any individual will require knowledge of the history of exposure to certain predisposing factors as well as an understanding of the ongoing immune response in that patient.

### 1.2.4 Factors that Influence Susceptibility to Tuberculosis

#### 1.2.4.1 Genetic factors

Susceptibility to TB is a multigenic trait. At present, few genes have been identified that are related to TB susceptibility/resistance. However, major

histocompatability complex- (MHC) genes and the *bcg* gene have been studied extensively in this context.

#### 1.2.4.1.1 Major Histocompatability Complex Genes

Numerous studies have shown that susceptibility/resistance to TB is partially attributable to MHC-proteins, both in animals and humans. This is not surprising as these proteins play such an integral role in T cell responses. In mouse models of intracellular infections, susceptibility and resistance are associated with particular MHC-(H-2) haplotypes (reviewed in Buschman et al., 1990). Studies in mice have shown that during infections with *M. tuberculosis*, H-$2^b$ appears to be associated with increased IFNγ production in response to mycobacterial antigens while H-$2^d$ is associated with lower IFNγ responses (Buschman et al., 1990). In a study using C57BL/6 mice (H-$2^b$), BALB/c (H-$2^d$) and MHC-congenic BALB.B10 (H-$2^b$) mice infected with *M. bovis* BCG, dramatic MHC-dependent differences in cytokine production by T cells were observed. Type 1 cyokines dominated the response of T cells of the H-$2^b$ mice, while type 2 cytokines, particularly IL-4, were produced in greater amounts by the cells of H-$2^d$ mice (Huygen et al., 1992). On the basis of median survival time after infection with *M. tuberculosis*, I-$A^b$ and $D^b$ alleles were associated with susceptibility while I-$A^k$ and $D^d$ alleles provided more resistance, establishing a role for both MHC-class I- and class II-restricted T cells (Apt et al., 1993). Finally, preliminary experiments suggest that MHC may be a significant factor in determining the dose response to BCG in MHC-congenic mice (Power and Bretscher, 1996).

Studies in humans have indicated a correlation of susceptibility or resistance to TB with particular MHC-alleles. Human lymphocyte antigen (HLA) -DR2 has been associated with an increased susceptibility to TB in several studies (Khomenko et al., 1990; Brahmajothi et al., 1991; Hill, 1998). In one study, HLA-B15 was also associated

with increased incidence of TB, while HLA-B27 and -DR3 were associated with increased resistance (Khomenko et al., 1990). HLA-DR4 has been linked with increased induration in response to skin test (Ottenhoff et al., 1986), indicating that this allele may be associated with enhanced cell-mediated responses.

#### 1.2.4.1.2 Nramp

A gene which is expressed exclusively in macrophages has been identified as a factor in determining resistance to several intracellular organisms including BCG (Potter et al., 1983), *Leishmania* sp. (Potter et al., 1983), *Toxoplasma gondii* (Blackwell et al., 1994b) and *Salmonella typhymurium* (Potter et al., 1983); hence, the gene has been given the various names *bcg*, *lsh*, and *ity* . Expression of this gene product is associated with an activated phenotype in the macrophage, resulting in a greater capacity to kill intracellular pathogens. The protein product of this gene is called the *natural resistance-associated macrophage protein* (Nramp) (Vidal et al., 1993). The gene was identified originally in inbred strains of mice. Different strains varied greatly in their ability to control an infection after intravenous (i.v.) injection of $10^4$ BCG mycobacteria (Pelletier et al., 1982). In some strains, the bacteria grew logarithmically in the spleen, liver and lungs of the infected mice while in other strains designated $BCG^r$, the number of BCG never rises higher than it is immediately after the initial infection. This 100- to 1000-fold difference in bacterial load was traced to a single autosomal gene mapping to the centromeric part of chromosome 1 (Skamene, 1994). Although the mechanism remains unclear, Nramp appears to be pleiotropic in its effects on macrophages, enhancing many aspects of their activation thereby enabling them to kill intracellular pathogens more effectively (Denis et al., 1988; Blackwell et al., 1994a). The protein associates with the phagosomal membrane and may be involved in transport of divalent cations. Although the direct functions of Nramp are exerted upon macrophages rather than T cells, the outcome of its effects can strongly influence acquired immunity, particularly in view of

the effect of dose on the immune response. Human homologues of the *bcg* gene have been identified and alleles are being tested for linkage to TB and leprosy (Skamene et al., 1998).

Of course the question of whether this gene controls susceptibility to virulent *M. tuberculosis* is of more interest than its control over an avirulent strain such as BCG. In a study using $BCG^s$ and $BCG^r$ mice, only infections with fewer than $10^3$ *M. tuberculosis* showed a detectable difference in mycobacterial growth in the lungs and spleens between the two strains of mice infected, with the lungs showing a more pronounced difference (Brown et al., 1995).

#### 1.2.4.1.3 Other Genes

Recessive mutations in type 1 cytokine genes that block their immune function have been associated with a high susceptibility to mycobacterial infections (Altare et al., 1998a; 1998b). Although these mutations are rare, they emphasize the importance of this pathway in providing immunity to *M. tuberculosis*. Thus, less dramatic allelic variations in genes that affect the ability of an individual to produce, or respond to type 1 cytokines are likely to influence susceptibility to TB infection. Such allelic variations have not been identified to date, but they most certainly exist.

### 1.2.4.2 Environment

The environment in which one lives has the potential to affect ones susceptibility to TB. Factors such as the rate of TB in the community, the presence of TB patients in the household, and crowded living conditions within the household or community have the potential to increase the level of exposure and thus infection with TB. However, as discussed previously, these factors are unlikely to determine whether an individual develops disease after infection.

One environmental factor that does affect the potential to develop disease upon infection is previous exposure to other mycobacteria, either those that exist in the environment or the BCG vaccine. As exposure to these cross-reacting bacteria has the potential to influence the subsequent immune response upon infection with *M. tuberculosis*, environmental bacteria can predetermine the effectiveness of the immune response to *M. tubeculosis* infection. This subject is discussed in greater detail in section 1.3.3.2.

### 1.2.5 Immunology of Tuberculosis

The immunology of mycobacterial infection continues to be the subject of much research. In the majority of individuals who are infected, the immune system is able to contain the primary infection, and the patient remains asymptomatic, most often for their entire life. In the remaining individuals, the disease progresses, presumably because the immune system fails to contain it, and the patient becomes symptomatic.

The question "what is effective immunity to *M. tuberculosis*?" is not easily answered. *M. tuberculosis* is a facultative intracellular organism, which spends most of its in vivo life within infected macrophages. Controlling intracellular pathogens such as *M. tuberculosis* is accomplished through the cellular arm of the immune system and both $CD4^+$ and $CD8^+$ cells are involved.

#### 1.2.5.1 Humoral Response

Many researchers have looked at the antibody response to mycobacterial infections in an effort to define the significance of this aspect of the immune response in immunity to mycobacteria. Although some early controversy existed, there is now little doubt that antibody does not play a role in protecting against such infections (reviewed in Rosenthal, 1980b). This is because the organisms of the *M. tuberculosis* complex are intracellular

organisms that live and grow within the protected environment of infected macrophages and are therefore not susceptible to the neutralizing effect of antibodies. Although some peptides of mycobacterial proteins may be expressed on the surface of infected cells in association with MHC-molecules, antibodies against these peptide/MHC-complexes probably would not be effective as there would likely be far too few antibodies bound to a cell to have an effect (Humphrey and Dourmashkin, 1969).

#### 1.2.5.2 Cell-Mediated Responses

##### 1.2.5.2.1 Delayed Type Hypersensitivity

Antigen-specific CD4+ cells producing type 1 cytokines mediate DTH which is considered an important immune mechanism for clearing intracellular pathogens including intracellular mycobacteria. DTH responses to mycobacterial antigens have been used for many years as an indicator of exposure to mycobacteria. In particular, skin tests performed with purified protein derivative (PPD) have been used as a diagnostic tool to determine if an individual has been exposed to *M. tuberculosis*. DTH responses to PPD have also been used to assess the effectiveness of BCG vaccination, and individuals who receive BCG are often given a skin test to determine if they have developed an immune response to the vaccine. Unfortunately, a positive skin test does not distinguish between exposure to *M. tuberculosis*, *M. bovis* BCG or another species of mycobacteria (WHO Tuberculosis Research Office, 1955a; 1955b). In animal studies, a positive skin test after vaccination with BCG is associated with increased protection against challenge with virulent *M. tuberculosis*, and there is a correlation in some studies between the degree of positivity (mm of induration) and resistance to disease (Tuberculosis Program - U. S. Public Health Service, 1955; Siebenmann and Barbara, 1974). However, in human trials of BCG vaccination, such a correlation does not exist (Tuberculosis Research Centre, 1999). Therefore, for humans, a more complete understanding of what constitutes

effective immunity to *M. tuberculosis* is necessary, as a simple evaluation of DTH responses by skin testing is not a predictor of protection.

#### 1.2.5.2.2 T helper Cells

Th1 cells play a dominant role in immunity to slow-growing intracellular organisms such as leishmania and mycobacteria, which selectively infect phagocytic cells of the macrophage lineage. These T cells produce the type 1 pro-inflammatory cytokines IFNγ and TNF. IFNγ and possibly TNF activate macrophages to enable them to kill intracellular mycobacteria (Nathan, 1986; Kaufmann, 1999;). These cytokines are also crucial for granuloma formation (Flynn et al., 1993; 1995; Kaufmann, 1999). Thus, induction of Th1 cells is critical in ensuring an effective immune response to mycobacterial infections. Th2 cells, on the other hand, are not important in conferring protection against these slowly replicating, predominantly intracellular pathogens. Moreover, induction of Th2 cells can be detrimental to the development of a protective response (Kaufmann, 1999).

#### 1.2.5.2.3 Cytotoxic T cells

Most research on the immunology of TB has focused on the $CD4^+$ T helper (Th) cell, although in recent years some evidence has suggested a prominent role for $CD8^+$ T cells. Studies in MHC-class I-deficient mice have shown an increased susceptibility to mycobacterial infection indicating that class I-restricted $CD8^+$ cells have a role to play in TB immunity (Flynn et al., 1992). The effector mechanisms of these cells are two-fold. They can produce immunostimulatory cytokines that stimulate phagocytic cell activation and they are also capable of recognizing and directly killing infected cells through various mechanisms. The relative importance of these various mechanisms in conferring immunity to TB is being investigated. In a mouse model of *M. tuberculosis* infection,

both $CD4^+$ and $CD8^+$ *M. tuberculosis*-specific T cells were found in the lung very early after i.v. infection with $2x10^5$ *M. tuberculosis*. Although $CD4^+$ cells dominated the response, a significant number of IFNγ-producing $CD8^+$ cells (approximately half the number of $CD4^+$ cells) were identified after in vitro stimulation with *M. tuberculosis* infected antigen-presenting cells (Serbina and Flynn, 1999). After aerosol infection of mice with *M. tuberculosis*, IFNγ-producing $CD4^+$ and $CD8^+$ T cells peaked in the lung tissue at 8 wk post infection (pi) and 4 wk pi in the lymph nodes draining the lung (Feng et al., 1999b). IFNγ-producing CD8+ cells have also been isolated in peripheral blood mononuclear cells (PBMC) after intranasal infection of cattle with *M. bovis* (Liebana et al., 1999).

The studies of Serbina, Feng, and Liebana have not examined the cytotoxic capabilities of the $CD8^+$ cells, but a previous study has shown a direct correlation between IFNγ-production and cytolytic activity in $CD8^+$ cells (Fabio et al., 1994). However, the relevance of the various cytolytic mechanisms of $CD8^+$ cells to protection against mycobacterial disease is ambiguous, at least in the early stages of infection. Perforin- and granzyme-knockout mice were found to be no more susceptible to *M. tuberculosis* in early stages of infection than normal mice (Cooper et al., 1997). Therefore, $CD8^+$ cells may be exerting their effects more through type 1 cytokine production than through direct lysis of infected cells in mycobacterial infections.

### 1.2.6 Immunologic Spectrum of Disease

Attempts have been made to define a spectrum of disease that correlates to immune function in TB patients. Tuberculosis patients were classified into two polar groups based upon the clinical presentation of the disease and radiological data (Lenzini et al., 1977). Patients classified as reactive (RR) had the least severe forms of disease while those with

acute miliary TB were designated as unreactive (UU). Two other intermediate groups were also defined, reactive intermediate (RI), and unreactive intermediate (UI). The groups were tested for various immunological, bacteriological, and histological parameters. All of the patients in the RR group were skin test-positive to PPD. In addition, these patients had no anti-PPD serum antibodies, reacted strongly in leukocyte migration-inhibition tests and had no mycobacteria present in either their sputum or their tissues. These patients had a response typical of that induced by Th1 cells, which seemed to confer some degree of protection. The patients classified as UU, however, were skin test-negative, leukocyte migration inhibition-negative and they had large numbers of mycobacteria both in their sputum and tissues as well as anti-PPD antibodies in their serum. The response in these patients was more typical of a Th2 response and appears to have given little protection from the disease. The RI and UI patients were between these two polar groups in a continuous immune spectrum as assessed by these parameters (Lenzini et al., 1977).

Bhatnagar et al. (1977) also proposed that a spectrum of immune responses after exposure to *M. tuberculosis* correlates with disease severity. Healthy, skin test-positive (presumably *M. tuberculosis*-exposed individuals) were included in this study. The results indicated a spectrum of disease, with healthy contacts demonstrating strong cell-mediated immunity (CMI), but little humoral response, seriously ill miliary TB patients exhibiting little cell-mediated immunity and increased humoral responses, and those with less severe forms of pulmonary TB lying midway in between the two extremes (Bhatnagar et al., 1977).

These studies suggested that individuals who develop a humoral response upon exposure to *M. tuberculosis* are more likely to develop disease and that the severity of the disease correlates with the prominence of the humoral response. These interpretations were reasonable based on what was known about effective immunity to mycobacteria. The subsequent discovery of distinct CD4+ subsets mediating distinct immune functions

and the understanding that these Th1 and Th2 subsets are cross- regulated provided an explanation of why the immune spectrum correlates with disease pathology. Individuals who generate a Th1 response upon infection with *M. tuberculosis* might be expected to contain the disease, whereas those who develop a Th2 response upon exposure would be expected to have severe disease because they would not be able to contain the organism. Patients who develop a mixed Th1/Th2 response would have varying degrees of disease depending on the relative strength of the Th1 component versus the Th2 component. A mixed Th1/Th2 response would be less effectual for clearing infection because the two arms of the immune response are cross regulated, and the Th2 cytokines such as IL-4 can inhibit the production and function of type 1 cytokines ( Mosmann and Coffmann, 1987; Lehn et al., 1989; Sher et al., 1991; 1992). In addition, IL-4 has been shown to act in TB lesions to increase the necrotic effect of TNF, thereby increasing damage and preventing healing of the lesion (Hernandez-Pando and Rook, 1994). This model suggests that TB patients with more severe disease should have a more prominent Th2 response, while those with less severe disease should have a more prominent Th1 response. Healthy individuals who have been infected with *M. tuberculosis* would have a Th1 response that protects them from disease.

In support of this model, Surcel et al. (1994) found a significant difference between the mean number of *M. tuberculosis*-specific IL-4-producing cells in the peripheral blood lymphocytes of a group of TB patients compared to the numbers in sensitized healthy controls. However, there was considerable variation among the patients with respect to this parameter, and so the difference between the groups was only apparent at the population level. Similarly in leprosy patients, IL-2 and IFNγ messenger RNA (mRNA) were generally higher in tuberculoid leprosy lesions, while mRNA for IL-4, IL-5 and interleukin 10 (IL-10) predominated in lepromatous leprosy lesions (Yamamura, 1991).

### 1.2.7 Histologic Spectrum of Disease

The idea of a histological spectrum of disease in TB patients stems from observations in leprosy patients (Ridley, 1974). Ridley compared the histological appearance of leprosy lesions with immunological parameters. The results indicated a spectrum of disease with two clearly defined poles. At one extreme are patients with lesions characterized by well defined epithelioid cell granulomas containing a significant number of lymphocytes and few, if any, acid-fast bacilli (AFB). At the other end of the spectrum are patients with loosely formed macrophage granulomas containing no epithelioid cells, few lymphocytes and very high numbers of AFB (Ridley, 1974). It is important to note that epithelioid cells are highly developed and activated cells of the macrophage lineage which are crucial for proper granuloma formation. Patients with the former type of disease, often called tuberculous leprosy, have a much better prognosis than those with the latter form, lepromatous leprosy.

A similar histological spectrum has been defined in TB patients (Ridley and Ridley, 1987). Upon histological examination of lesions, patients were divided into six groups based on the cell types, organization, and necrosis observed in tuberculous granuloma. The lesions of individuals at one pole consisted of highly organized granuloma in which mature epithelioid cells predominated and in which there was little or no sign of necrosis and few bacilli. This type of lesion is seen predominantly in the cutaneous form of TB known as lupus vulgaris. These lesions correspond histologically to those seen in the tuberculous leprosy patients described previously. At the other end of the spectrum were patients with fatal disseminated disease. The lesions in these cases showed extensive basophilic or eosinophilic necrosis with many AFB present. In lesions of this group, macrophages represented the predominant granuloma cell type, but these were few in number and the granuloma lacked defined structure (Ridley and Ridley, 1987). The majority of patients, however, were found to be between the two poles,

having varying degrees of illness that correlated with the ability of the immune response to contain the bacilli within the granuloma.

It appears that there is a spectrum of disease ranging from healthy sensitized contacts who have a strong Th1 response and are able to contain the pathogen to those individuals with severe disseminated disease who have a purely Th2 response and are, therefore, unable to control the pathogen. However, in TB patients, this pattern does not take into account all individuals since, in some studies, not all of the patients fit into a spectrum so defined and an association between a Th2 response and disease is only apparent as a trend in the population. In particular, in some patients with TB, the immune response does not show a Th2 component. Consider the lupus vulgaris patients in the study by Ridley and Ridley (1987). These patients mounted classical Th1 responses against the organism, with well-defined granuloma containing few bacilli, and yet they were unable to control the infection effectively as clinical manifestations of the disease continued. These individuals do not fit into the proposed immunological spectrum as it is currently defined.

### 1.2.8 A Model Explaining Tuberculosis Immunopathology

#### 1.2.8.1 Two Types of Immunopathology in Tuberculosis

One model of TB immunopathology suggests that the disease is the result of an individual developing a Th2 response upon exposure to *M. tuberculosis*. I feel that although this model is correct in many respects, it is insufficient to describe the cause of progression of TB in many patients, specifically, those who do not fit into the immunological/disease spectrum as it is currently perceived. Dr. Bretscher, myself and some of the other members of the Saskatchewan Research Centre for Elimination of Tuberculosis propose a new spectrum as shown in Figure 1-1. According to this model, *M. tuberculosis* infected individuals develop disease as a result of two distinct types of

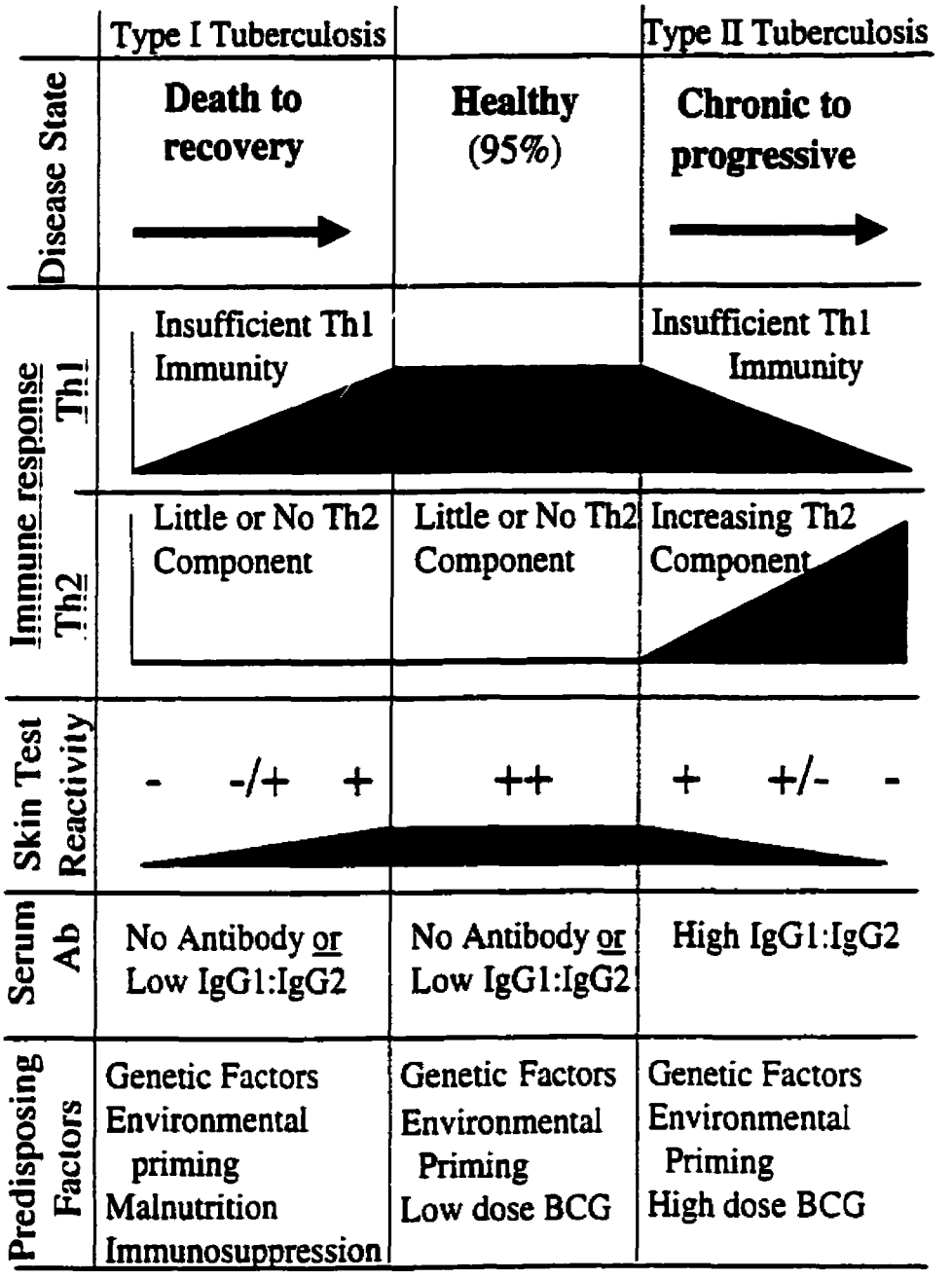



**Figure 1-1** A model for immunopathology of tuberculosis. Tuberculosis is the result of an ineffective immune response against *M. tuberculosis*. Disease may result if the immune response is an appropriate Th1 response but of insufficient strength to control the infection (Type I tuberculosis) or if the immune response has a Th2 component that interferes with the Th1 response (Type II tuberculosis). The predicted immune response of the respective groups as well as some of the predisposing factors for each type of disease are shown.

failure of the immune system which lead to distinct immunopathological states. The majority of natural infections will result in the induction of an appropriate Th1 /cell-mediated immune response that is able to contain the pathogen without development of clinical disease (Figure 1.1, center). In a small percentage of infections, a Th1 response develops, but for some reason this immune response is insufficient to contain the infection and clinical disease results (Figure 1.1, left). In these individuals, the severity of the disease depends entirely on the strength of the Th1 response. We expect that these individuals have Th1 responses to mycobacterial antigen, but responses will not have a significant Th2 component. If anti-mycobacterial antibodies are generated, the immunoglobulin-γ-2 (IgG2) subclass which are associated with a Th1 response (Kitani and Strober, 1993; Kawano and Yata, 1994), will be prominent. We refer to such individuals as Type I TB patients. In another small group of infections, the immune response is not entirely a Th1 response, having a significant Th2 component (Figure 1.1, right). In these individuals, the severity of the disease depends upon the relative proportions of the Th1/Th2 components. These people will have varying numbers of Th2 type cells. We refer to these individuals as Type II TB patients. Discriminating between these two types of patients will be crucial to understanding the relationship between the pathogen and the host immune response in TB as well as in other diseases. Measuring levels of various immunoglobulin subclasses may provide a means of discriminating between these groups.

It is important to clarify the difference between insufficient Th1 immunity to control the TB infection and an immune response that has a detrimental Th2 component. I have previously discussed the clinical and immunological evidence that some TB patients may have developed the disease because they have an immune response that has a Th2 component, i.e., Type II patients. The patients that we would classify as Type I patients have an ongoing immune response to *M. tuberculosis* and we maintain that they have the appropriate type of immune response. Why then are they unable to clear the infection? I

hypothesize that either the bacterial load in the ongoing infection is too great to be cleared by the established immune response, even though the same immune response could clear a lesser infection, or alternatively, that prior exposure to environmental mycobacteria results in immune suppression that prevents a strong Th1 response from developing.

The Koch phenomenon (Koch, 1890) represents an experimental model of the former mechanism. Guinea pigs injected on one flank with virulent *M. tuberculosis* develop a lesion. However, after several wk, if these same animals are given a second injection of an equal number of bacteria on the other flank, they are able to wall off and heal the infection at the second site, while the infection at the primary site progresses. This suggests that these animals have sufficient immunity to control an infection with the relatively large number of organisms given in the secondary infection, but that this immunity is insufficient to control an established infection. Similar observations have been reported in models of tumor growth (North, 1985). In mice injected with a given number of P815 tumor cells on one flank, a solid tumor develops; however, while the primary tumor continues to grow, the mice are able to eliminate a second injection of the same given number of tumor cells at another site. The initial inoculum induces sufficient immunity to eliminate the second inoculum, but not the established tumor. This is referred to as concomitant immunity (North, 1985). Why can't the immune system clear the infection at the primary site? One obvious reason is that in the time it takes to establish the immune response, the organism (or tumor cell inoculum) grows uninhibited and by the time the immune response is instituted, the tumor or infection is too large to be effectively cleared. However, a solid tumor is very different from an intracellular organism infecting many individual cells. In TB infections, there are at least two explanations for why the immune response is not effective despite concomitant immunity.

1. *M. tuberculosis* has been shown to have several inhibitory affects on the response of immune cells (Hussain et al., 1999; Ting et al., 1999). Thus, in the time required to establish an immune response to the organism, immune cells responding locally to the infection may become subject to suppressive actions of the bacteria before they can be fully activated. Cells induced at a distance from the site of infection would still be functional and thus capable of inhibiting infection at a second site.

2. The immune response itself may be partially responsible for its ineffectiveness in clearing a TB infection. The immune response to TB often results in granuloma formation. The function of the granuloma is to surround mycobacteria infected cells and trap them within to contain the infection. However, the formation of a granuloma may actually prevent efficient immune clearance of the pathogen as it may remain viable within the caseous center of the granuloma for many years.

Immune suppression generated after exposure to environmental mycobacteria offers an alternative reason why an appropriate Th1 response to *M. tuberculosis* is induced but remains ineffective in controlling the disease. TB patients who have positive skin sensitivity to *M. tuberculosis* can have reduced or absent responses to environmental saprophytic mycobacteria (Kardjito et al., 1986). One might consider that the immune response to the environmental saprophytic mycobacteria in these patients is a predominant Th2 response and so no skin reactivity to these bacteria is observed. However, as demonstrated here with BCG and as Rook has reported with killed *M. vaccae* (Hernandez-Pando et al., 1997), mycobacterial immunization usually results in either a Th1 response or a mixed Th1/Th2 response depending on the dose administered. Therefore, it seems unlikely that there would be a large number of individuals with a predominantly Th2 response to environmental mycobacteria, and a mixed Th1/Th2 response to *M. tuberculosis* as there is considerable cross-reactivity between mycobacterial species. It is perhaps reasonable to assume that the individuals who have reduced or absent skin-test sensitivity to environmental mycobacteria have a suppressed

response to the environmental mycobacteria and that the reactivity observed on skin tests with *M. tuberculosis* PPD is directed against *M. tuberculosis*-specific antigens. The lack of immunity to environmental mycobacteria observed in some individuals could be explained by a mechanism of oral tolerance. Exposure to mycobacteria in the gut may induce T cells that produce such factors as transforming growth factor β (TGF-β) that suppress the systemic immune response to mycobacteria. Studies in leprosy patients and their contacts support such a mechanism of active suppression. In the leprosy patients, sonicates of fast growing mycobacteria suppressed the local response to slow growing mycobacteria (Nye et al., 1983).

If there is some degree of unresponsiveness to environmental mycobacteria due to environmental priming, then it is reasonable to assume that the responsible mechanisms will affect the subsequent response to *M. tuberculosis* due to cross-reactivity between the mycobacterial species. Thus, a down-regulated response to environmental mycobacteria may prevent these individuals from mounting an optimal immune response to *M. tuberculosis*. This may represent a mechanism through which the immune system of some individuals is prevented from mounting an optimal Th1 response upon exposure to *M. tuberculosis* and thus these individuals would develop "Type I" tuberculosis.

#### 1.2.8.2 Predictions from the Model

The model makes several predictions. Firstly, the model predicts that some individuals who are infected with TB and who develop clinical disease will recover without the aid of chemotherapeutic agents. These individuals are expected to be those with an initially insufficient Th1 response that increases with time so that it can eventually contain the infection. Epidemiological studies conducted in the pre-chemotherapeutic era indicate that this is indeed the case. Many of the patients who were admitted to sanatoria with TB eventually recovered fully from the disease without the benefit of antibiotics

(Brown, 1919; Rutledge and Crouch, 1919). Presumably, the immune system of these individuals was eventually able to control the infection.

Secondly, some TB patients are expected to benefit from immune interventions designed to boost their immune response to TB. Such treatment could be a valuable adjunct to antibiotic treatment. The patients benefiting from such interventions would be those suffering from Type I TB, in which boosting the immune response would lead to an increase in the appropriate Th1 response required to clear the infection. Those with a Th2 response would not benefit from administering antigen as immunotherapy as this would be expected to only lead to a stronger Th2 component that would not help in clearing the infection and may actually exacerbate the disease due to increased immune-mediated tissue damage and necrosis. Immunotherapy for TB has not been attempted frequently as history records that Robert Koch's initial attempts at immunotherapy were disastrous in some cases. Koch used crude tuberculin preparations to treat patients suffering from various forms of tuberculous disease and also provided this material to other physicians for similar trials (Koch, 1890; Koch, 1891). Historical discussions of these studies indicate that the treatment was an utter failure as many individuals died rapidly as a result of the tuberculin treatments (Virchow, 1891). However, what is not discussed in historical references to this work is the fact that, after tuberculin treatment, many TB patients recovered fully (Coghill, 1891).

I predict that the latter were most likely those with the Type I form of the disease, while the former suffered from Type II tuberculosis. There is no direct proof from these studies that the patients who benefited from the tuberculin therapy had Th1 immunity while those patients whose conditions worsened had Th2 immunity, but descriptions of the individual cases support this hypothesis. Patients with one clinical form of the disease were almost invariably cured after treatment with tuberculin (Koch, 1890; Hospital for Diseases of the Chest, 1891). These patients suffered from a cutaneous form of *M. tuberculosis* infection commonly referred to as lupus vulgaris. Modern histological

examination of lupus vulgaris cases demonstrate that the lesions observed in these patients are associated with a Th1 response. These lesions are characterized by well-structured granuloma containing predominantly epithelioid cells (Ridley and Ridley, 1987). It seems likely in retrospect that these patients suffered from the disease because their immune response was not strong enough to control the infection even though they mounted an appropriate type of immune response. This surmise explains why boosting the immune response led to clearance of the infection. Lupus vulgaris patients were not the only ones that benefited from the treatment as numerous pulmonary TB patients also recovered after tuberculin injections (Coghill, 1891). The patients who benefited from treatment were those with less severe forms of disease and it is likely that some of these patients would have recovered eventually even without treatment or perhaps they would have lived for many years with chronic disease. Many other patients suffered greatly as a result of the treatment with tuberculin (Virchow, 1891; Coghill, 1895). Because of this and a lack of understanding of the pathology of the disease, all tuberculin treatments were discontinued and the idea of immunotherapy for TB has become stigmatized. It is interesting to note that many of the patients suffered as a result of improper use of tuberculin therapy - the use of very high doses and use in patients with severe disease (Coghill, 1895) - practices contraindicated by Koch himself (Koch, 1890).

If this model of TB in humans is correct, there will be considerable implications for vaccination against and treatment of the disease. For vaccination purposes, an immunizing regimen that induces only Th1 responses will be beneficial to individuals prone to develop either Type I or Type II tuberculosis. For individuals who would develop Type I TB upon exposure to *M. tuberculosis*, any immunization which boosts Th1 immunity would be beneficial as it may allow them to clear the infection more readily. For those patients who would develop a Th2 response and thus Type II tuberculosis when exposed to *M. tuberculosis*, a vaccination strategy that allows them to

develop a Th1 imprint and hence a Th1 response upon exposure probably would prevent the disease.

For treatment of disease, understanding the implications for patients with different types of TB is important. As I have suggested previously, early treatments with tuberculin may have helped patients with Type I TB by boosting their Th1 response. Therefore, patients with this type of disease may benefit from immunotherapy as an adjunct to chemotherapy. Those with a Th2 response would not benefit from antigen therapy as it would probably boost their Th2 immunity and perhaps exacerbate disease. Defining parameters that can distinguish between these two groups is a priority for the Saskatchewan Research Centre for the Elimination of Tuberculosis.

## 1.3 Bacille Calmette Guerin (BCG)

### 1.3.1 Early History

BCG is an attenuated strain of *M. bovis* that was developed over a period of 13 years (1908-1921) by two French scientists, Alain Calmette and Camille Guerin working at the Pasteur Institute in Lille, France (Guerin, 1980). Using bovine bile as an emulsifier to prevent clumping of the BCG, they soon discovered that this treatment reduced the virulence of the bacillus. They continued to passage this bacillus every three weeks, checking its virulence in animals until, after 13 years and 231 passages, it was completely non-pathogenic in animals.

On July 18, 1921, the first dose of BCG was administered to a human infant by Dr. Benjamin Weill-Halle in collaboration with Calmette and Guerin (Weill-Halle and Rosenthal, 1980). The child was chosen for this trial because he was to be raised by his tuberculous grandmother as his mother had died of TB in child-birth. It was felt that the child was also doomed to die of the disease. The infant was fed three doses of the vaccine in his first two weeks and remained free from TB for life. This first human trial did not demonstrate the efficacy of the BCG vaccine as the child may have never

developed TB without it; rather its significance is that it demonstrated the safety of the vaccine for humans. Since the League of Nations certified BCG as safe for human use in 1928, more than 2.5 billion people have been vaccinated with the BCG vaccine (Stover et al., 1991).

### 1.3.2 Efficacy studies in Humans

By 1948, more than 10 million people had been vaccinated with BCG. Despite this, reports of the protection provided by the vaccine were almost entirely anecdotal. It was not until the late 1940s that reports of properly controlled studies became available. An extensive and excellent review of the results of BCG vaccination trials conducted before 1980 is included in BCG Vaccine: Tuberculosis-Cancer (Rosenthal, 1980a). There has been tremendous variability in the outcome of BCG vaccination trials. Early studies in North American Indians conducted by Ferguson in Canada (1928) and Aronson in the United States (1958) found a similar rate of protection of approximately 80%. Ferguson also found a high rate of protection among nursing students in Saskatchewan (1955). A study conducted by Hart and Sutherland (1977) found a protection rate of 78% among British schoolchildren. However, many other studies have shown far lower levels of protection with the BCG vaccine (Comstock and Webster, 1969; Comstock et al., 1976). The Madras trial conducted in South India by the WHO, reported no overall effect of BCG vaccination (Tuberculosis Prevention Trial, 1980; Tuberculosis Research Centre, 1999). A meta-analysis of the published studies on BCG efficacy reported an overall protective effect of 50% (Colditz et al., 1994), although this number is rather meaningless as it does not predict the potential efficacy of vaccination against the disease in any one population. The variability in efficacy of the BCG vaccine found in various trials is likely to reflect a number of factors that affect the immune response to BCG. A description of these factors and their probable effect on the outcome of BCG vaccination follow.

### 1.3.3 Variables Affecting the Efficacy of BCG in Humans

#### 1.3.3.1 Genetic Factors

In general, Caucasians appear to be more resistant to TB than Africans, Asians and North American Indians. This observation is thought to be due to the fact that Caucasians have been exposed to the bacterium for many generations and, therefore, this population has developed a relatively high level of resistance due to the process of natural selection (Cummins, 1920; Ferguson, 1955). Elimination of individuals from a population who have genes or alleles which predispose them to developing disease or more serious forms of disease, results in a more resistant population. The longer the population has been exposed to *M. tuberculosis*, the greater the resistance to TB. This selection process is likely to affect the response to the closely related *M. bovis* BCG. This topic is discussed in further detail in section 1.2.4.1.

#### 1.3.3.2 Environment

The primary environmental factor that is likely to affect the outcome of BCG vaccination is the presence of other mycobacterial species living in the environment. The mycobacteria represent a diverse group of organisms. Many species are commonly found in soil or water, or as non-pathogenic organisms in animals and in man. Most individuals living in areas where such mycobacteria are present, will have positive skin-test responses to tuberculins made from these organisms, indicating that the organisms are capable of inducing an immune response in humans. These organisms have been shown to confer some degree of immune protection from virulent mycobacteria in animal studies (Palmer and Long, 1966). Originally, Palmer and Long (1966) suggested that exposure to environmental mycobacteria is one reason why some BCG studies indicate a much lower efficacy level than others. They hypothesized that if a population exposed to cross-reacting environmental mycobacteria is subsequently given BCG, then the level of protection detected in the study would be only the additional protection provided by the

BCG vaccination. The additional level of protection provided by BCG vaccination in areas where environmental mycobacteria are prevalent will be somewhat less than that in areas where BCG vaccination is the only means of protection. Accordingly, studies conducted in areas where environmental mycobacteria are prevalent, may seriously underestimate the protective value of the vaccine. In one study, monocytes from Mantoux-negative patients from the Chingleput region of India inhibited intracellular growth of *M. microti* more efficiently than monocytes from children in London. After BCG vaccination, monocytes from the London population had increased bacteriostatic activity, while those from Chingleput did not (Cheng et al., 1993). The authors suggest that exposure of the subjects from India to atypical mycobacteria may be responsible for the observed results.

Stanford et al. (1981) carried this hypothesis one step further. They proposed that the influence of environmental mycobacteria may not always be beneficial and suggested that the immune response generated by exposure to such mycobacteria will vary depending on the species to which one is exposed and perhaps on the conditions of exposure. In some cases, a protective immune response may be generated, while in other cases a detrimental immune response is the result. Thus, in locations where environmental mycobacteria are prevalent, a beneficial or detrimental immune response may be generated before BCG vaccination and subsequent exposure to BCG simply boosts the established response. Once again, according to this model, there will be little effect of BCG vaccination in areas where environmental mycobacteria are common.

Subsequent experiments suggested that a beneficial response was a purely Th1 response, while the detrimental response was one with a Th2 component (Hernandez-Pando and Rook, 1994; Hernandez-Pando et al., 1997). The evidence suggested that in a purely Th1 response, TNF acts as a pro-inflammatory, Th1 cytokine, but if even a small amount of IL-4 is present, then the lesion becomes necrotic. Hernandez-Pando et al. (1994, 1997) hypothesized that IL-4 in the lesion sensitizes the tissue to the necrotizing

effects of TNF, thereby leading to destruction of surrounding tissue and chronic disease (Hernandez-Pando and Rook, 1994; Hernandez-Pando et al., 1997).

#### 1.3.3.3 Vaccination Parameters

Vaccination parameters such as route of injection, dose of BCG administered and the strain of BCG used to vaccinate are also determinants of the immune response to BCG vaccination. However, the influence of varying these parameters can be determined experimentally and controlled in vaccination procedures so that the response is optimal.

##### 1.3.3.3.1 Route of Injection

The three routes of administration that have been most commonly used for vaccinating humans with BCG are oral, intradermal (i.d.) and transcutaneous. The oral route of administration was the first method used to administer BCG, in the belief that in the first two weeks of infancy, the intestinal mucosa were more permissive to the transport of microbes, allowing for more effective vaccination (Weill-Halle and Rosenthal, 1980). It appears that the organism is able to gain access to the blood in infants who are immunized orally (Weill-Halle and Rosenthal, 1980). Indeed, using the oral method of administration, younger children had higher tuberculin reactivity than those who were older at the time of vaccination (Weill-Halle and Rosenthal, 1980). The oral route continued to be used in many countries for some time. In particular, Brazil had used the oral route for many years and advocated the use of higher doses and multiple administrations. However, the oral method has been replaced by the i.d. route even in Brazil for reasons of efficacy and the desire to vaccinate individuals who were beyond infancy. Furthermore, with this method, discrimination of individuals who have been vaccinated from those who have been exposed to *M. tuberculosis* is not possible, due to the lack of the BCG scar after oral vaccination.

The transcutaneous method of vaccination includes the multiple puncture method and the scarification method. The multiple puncture method consists of applying a suspension of BCG vaccine to the skin surface and inserting a needle or multiple needles through the suspension to a depth of 2-3 mm into the skin. This method was pioneered by Rosenthal and is thought to be as effective as i.d. injection with less serious side effects (Weill-Halle and Rosenthal, 1980). It is the only method that is licensed for use in the United States (Lowry et al., 1998). A modification of this method, commonly referred to as scarification, involves placing a drop of BCG vaccine on the skin of the patient and scratching the surface of the skin with the point of a lancet. Because the vaccine is applied to the surface of the skin by the transcutaneous method and a needle is passed through the applied vaccine, only a small fraction of the vaccine will effectively induce an immune response. Estimating the exact number of organisms in the vaccine dose is impossible.

The i.d. route of administration of BCG is the most commonly used method and is recommended by the World Health Organization. In Canada, BCG vaccination is carried out using reconstituted freeze-dried preparations of BCG given intradermally. Approximately $10^7$ colony forming units (cfu) is administered to adults and half that number to infants in 100 µl and 50 µl volumes, respectively (Hoeppner, 2000). This is the preferred method of vaccination because of its efficacy and reproducibility in inducing an immune response as assessed by positive skin test.

#### 1.3.3.3.2 Strain of BCG Used for Vaccination

The strain of BCG that is used has a significant impact on the outcome of the immune response and/or the degree of protection after vaccination. Lagranderie et al. (1996) characterized the immune response and protective activity of five BCG strains. They report significant differences between the various strains in their ability to induce an immune response and provide protection from challenge in mice. All these strains came

from the same source initially, but repeated passage and different handling conditions have no doubt resulted in substrains with different growth properties. Behr and Small (1999) have characterized the genetic makeup of various strains and have found great differences between strains that probably account for the differences in efficacy between strains

#### 1.3.3.3.3 Age at Time of Vaccination

The age of the patient at the time of vaccination has an influence on the effects of BCG vaccination. Individuals who live in areas where they are likely to be exposed to environmental mycobacteria are more likely to be sensitized to environmental mycobacteria as they age (WHO Tuberculosis Research Office, 1955c). In some cases, this exposure may have an immunizing effect similar to BCG vaccination whereas in others, such exposure will result in an inappropriate response (see section 1.3.3.2). As this exposure occurs in an uncontrolled manner, the outcome of such exposure will vary from area to area and individual to individual, and certainly a desirable immune response will not occur in all individuals who are exposed. Vaccination with BCG at an early age, preferably in the neonate before the detrimental effects of environmental exposure occur, will be more likely to give a desirable response to BCG.

#### 1.3.3.3.4 Dose of Vaccine

Rarely has the effect of BCG dose been examined in human studies. Animal studies have often reported that higher doses of BCG provide more effective protection against subsequent challenge (discussed below), therefore, the highest tolerable dose is used for human vaccination. When more than one dose of BCG has been employed in vaccination studies, the dose range has been small, within ten fold of the highest dose used (Tuberculosis Prevention Trial, 1980; Tuberculosis Research Centre, 1999). In the context of the proposed model, such a dose may be still too high to discern a significant

difference from the standard dose. In one study which purports to look at low dose vaccination in humans (Lowry et al., 1998), a dose of BCG which is 1000-fold lower than the standard dose was administered to human subjects and compared with the standard dose. The authors found little evidence of an immune response to the low dose of BCG as assessed by IFNγ-production or proliferation by peripheral blood mononuclear cells at 16 wk and 1 yr pi. However, such responses were minimal in the individuals who received the higher doses, and as the vaccine was administered by the multiple puncture method, only a small portion of the dose would have gained entry into the tissue. By this method, even at the highest dose, the actual number of bacilli that interact with the immune system is relatively low. It has been reported that the multiple puncture method is far less effective at inducing IFNγ production in PBMC than the i.d. route of vaccination (Kemp et al., 1996), even when the "dose" used for transcutaneous immunization was 50 times that used for immunization by the i.d. route. It is interesting in this regard that proponents of this method of vaccination purport that it is highly effective at inducing tuberculin conversion, but with fewer side effects (Weill-Halle and Rosenthal, 1980). Perhaps in the study by Lowry et al. (1998), the few BCG bacilli introduced into the skin at the lowest doses administered, were cleared by the innate immune system before inducing an immune response. Alternatively, the immune response may have taken longer than 16 weeks to develop after immunization with the lower dose.

### 1.3.4 Animal studies with BCG

A large number of animal studies have examined the efficacy of BCG vaccination using challenge with virulent strains of *M. tuberculosis* to assess protection (reviewed in Rosenthal, 1980c; Smith, 1985). Trying to make sense of the vast amount of literature on the subject is difficult primarily due to the lack of continuity in experimental approach

between the various studies. I will not attempt to give a review of the results reported in the various papers on the subject. This has been done previously and in an extensive manner that I am sure I could not improve upon (Rosenthal, 1980c; Smith, 1985). However, one thing is apparent from reading the literature – BCG vaccination can provide protection to animals against subsequent challenge with virulent mycobacteria. None the less, there are a very large number of variables that must be considered in such studies, all of which may have significant impact on the outcome, or the conclusions drawn from these studies. Without taking all of these variables into consideration, it is possible to draw incorrect conclusions about the potential for a candidate vaccine. For this reason, I feel that it is beneficial to be aware of the importance of these variables to consider and how they may affect the outcome of the BCG trials.

#### 1.3.4.1 The Strain of Animal

Some strains of mice are more resistant to certain infections than others and this correlates to the type of immune response which is generated. BALB/c mice are more prone to develop Th2 responses to most antigens than other strains such as C57BL. Therefore, they are more likely to develop disease when infected with organisms which are only cleared effectively by a Th1 response. These differences are due to genetic variations within species, many of which influence the immune response that the animal develops upon exposure to the organism. Thus, it is important to understand the chosen animal model, its relative susceptibility to the infecting organism and the immune mechanisms that determine this susceptibility, for what constitutes protection in one strain of mouse may not lead to protection in another strain.

#### 1.3.4.2 The Strain of BCG

As discussed previously, the strain of BCG that is used may have profound effects on the outcome of any such study, whether in humans or in animals.

#### 1.3.4.3 The Route of Vaccination

Numerous routes of vaccination have been employed in animal studies, including oral, subcutaneous (s.c.), intraperitoneal (i.p.), i.d., i.v., transcutaneous and aerosol. It is certain that the route of administration will affect the outcome of vaccination, under a single set of conditions. Antigens are certainly more effective immunogens by some routes of administration than others (Lagrange et al., 1974). However, I believe that it is possible to achieve a full range of immune responses, regardless of the parenteral route of administration, simply by varying the conditions of immunization.

#### 1.3.4.4 The Interval between Vaccination and Challenge

In experiments that compare protection rates of various vaccines, it is important to take this variable into account. Most studies of protection have looked at the protective efficacy of a BCG vaccine by challenging relatively shortly after vaccination. In such cases, it may be possible that the immune response has not reached its peak, or that the challenge could interfere with the developing immune response. In both cases, protection could be greater if the challenge took place at a later time point. To ensure that these problems do not occur, it is important to perform kinetic studies that look at both the immune response to the vaccine as well as the protective effect.

#### 1.3.4.5 The Dose of Vaccine Administered

Previous studies examined the effect of dose on the protective efficacy of BCG vaccination. In general, these studies are lacking in that they have not taken into consideration many of the variables discussed above. For instance, in studies that compare various doses of BCG, the higher doses may induce a more rapid response than lower doses, however, the lower doses, in time, may generate a more appropriate and hence more effective response as observed in other systems (Salvin, 1958; Parish, 1972;

Bretscher et al., 1992). If the interval between vaccination and challenge is too short, i.e., before the lower dose can establish its peak response, then the lower dose will appear less effective than its potential (Smith, 1985). This situation has been seen in studies of leishmania infection (Bretscher et al., 1992). Once again, kinetic studies that examine the immune response at various time points after infection are important to distinguish the effectiveness of various vaccine doses.

#### 1.3.4.6 Other Factors

Other factors that will influence the outcome of the protection assays include the virulence of the challenge strain, the challenge dose administered and route of administration, as well as the method used to assess protection, i.e., survival time, bacterial burden or pathology.

### 1.3.5 The Immune Response to BCG Vaccination

Surprisingly, there is scant information on the immune response induced by BCG vaccination considering the degree to which it has been studied as a vaccine for TB. Most studies of BCG vaccination have looked at the protective effect of vaccination in preventing TB morbidity and mortality. Few have assessed classical measures of the immune response, and most of these have assessed only one aspect of the immune response generated, i.e., antibody or cytotoxic T lymphocyte (CTL) responses.

#### 1.3.5.1 The Cellular Immune Response to BCG Vaccination

In South India, where BCG vaccination failed in the last WHO BCG trial (Tuberculosis Prevention Trial, 1980; Tuberculosis Research Centre, 1999), various parameters of the immune response were evaluated both before and after intradermal vaccination with the standard dose of BCG. Individuals who were skin test- (Mantoux) negative before vaccination became skin test-positive; however, this did not correlate with

an increase in INFγ-production by PBMC (Das et al., 1998). In another study, Kemp (1996) found that $2x10^6$ BCG given by the i.d. route not only induced strong DTH responses at 2 months after vaccination, but *M. tuberculosis*-specific proliferation and IFNγ-production by PBMC were also induced. PBMC from healthy donors exposed to BCG in vitro demonstrated a sequential expression over time of cytokines from the Th1 type to the Th2 type. Initial cytokine production in the first 4-5 days after stimulation included IFNγ, TNF, and IL-2. At later times, Th2 cytokines including IL-4, IL-5, and IL-10 increased while Th1 cytokines declined (Sander et al., 1995). Smith et al. (1999) looked at the $CD8^+$ T cell response in humans after vaccination with BCG. They found that these cells produced IFNγ and TNF when stimulated with mycobacterial antigen. The cells also contained perforin granules and were capable of cytolytic activity.

Mice deficient in $CD4^+$ or $CD8^+$ T cells had similar levels of Th1 cytokines compared to normal controls, and were able to control pulmonary infection after intratracheal inoculation with $5x10^5$ BCG. In contrast, interleukin $12^{-/-}$ mice had an impaired ability to control the infection as assessed by histological examination of lung tissue (Xing et al., 1998). The results suggest that BCG induces both CD4+ and CD8+ cells and that they play an overlapping role in providing protection against BCG. The important role of 12 IL-12 in mycobacterial immunity is also highlighted. Five different strains of BCG induced IFNγ responses in lymph node cells of BALB/c mice immunized by the s.c. route (Lagranderie et al., 1996). IL-2 was also induced and the amount of both cytokines produced varied with the strain of BCG used for immunization. BCG changed the cytokine profile of $CD4^-$ natural killer T cells in the livers of mice immunized with BCG. These cells tend to produce IL-4, but after vaccination the ratio of IFNγ- to IL-4-producing cells was greatly increased. The authors suggested that BCG altered

cytokine expression by $CD4^+$ T cells by IL-12 induction in macrophages (Emoto et al., 1999). $CD8^+$ CTL specific for the 60 kDa mycobacterial heat shock protein (hsp60) were induced in C57BL/6 mice by i.v. immunization with $5x10^6$ BCG (Zugel and Kaufmann, 1997). $CD8^+$ T cells from BCG immunized donors have cytolytic activity against BCG- or PPD-pulsed autologous target cells (Smith et al., 1999).

#### 1.3.5.2 The Humoral Response to BCG Vaccination

Turneer et al. (1998) looked at anti-PPD immunoglobulin responses in BCG vaccinated individuals and TB patients. They found that BCG vaccination (approx. $10^5$ BCG intradermally) induced slight antibody responses in the majority of vaccinated individuals, and that the levels of antibody induced were similar to the levels seen in TB patients. Beyazova (1995) found that anti-PPD antibody levels in infants immunized (route and dose unknown) with BCG in their first two months after birth showed a steady but gradual increase in anti-PPD antibodies until the end of the experiment at 15 months after immunization. No age-matched controls were included in this study, so there is no proof that the change in titer is actually due to BCG vaccination. Similarly, antibodies to *M. bovis* BCG antigen A-60, the main component of old tuberculin, increased in infants over a six month post-vaccination period of observation (Rota et al., 1994). Individuals vaccinated with $1\text{-}5x10^5$ viable units of BCG by the i.d. route demonstrated a seroconversion rate of 6/21 to a purified protein antigen of BCG (P64) six wk after immunization (Drowart et al., 1992).

## 1.4 Recombinant BCG Vaccination

The possibility of using recombinant BCG as a vehicle for immunizing individuals against a variety of infectious organisms has been an area of interest for researchers for many reasons as described (Stover et al., 1991):

1. BCG is the most widely used vaccine in the world, with between 2.5 and 3 billion people having received the vaccine since it was developed.
2. It is a relatively safe vaccine with a low level of complications.
3. It can be administered any time after birth.
4. It is a live vaccine so a single dose can provide long term immunity.
5. Mycobacteria in general have immunopotentiating effects, which ensure a strong immune response.
6. BCG vaccine is relatively stable.
7. The vaccine is relatively inexpensive to produce.
8. BCG has the capacity to carry a large amount of foreign DNA offering the opportunity to produce multivalent vaccines.

The use of recombinant mycobacteria, particularly BCG, expressing foreign DNA as vaccine vectors is a relatively new concept. The first reports of mycobacteria expressing foreign DNA were made in 1987 (Jacobs et al., 1987). This group used chimeras containing bacteriophage DNA into which an *E. coli* cosmid had been inserted. This *phasmid* replicates as a plasmid in *E. coli* and as a phage in mycobacteria. This shuttle vector permitted the introduction of foreign DNA into *M. smegmatis* and BCG. In 1988, this group was able to express the kanamycin resistance gene as a selectable marker in a similar shuttle phasmid (Snapper et al., 1988).

Since then, several other techniques have been developed to introduce foreign genes into mycobacteria. These comprise the use of bacteriophages (Hatfull et al., 1994; Lee et al., 1991) and transposons (Gicquel, 1994), but the most common method is the use of hybrid plasmids designed to replicate both in mycobacteria and also in *E. coli*. Such plasmids allow gene insertion and manipulation to be performed using the fast growing *E. coli*, before introducing the plasmid into mycobacterial species by electroporation. The *M. fortuitum* plasmid pAL5000 (Ranes et al., 1990; Rauzier et al.,

1988), a plasmid capable of replicating in other mycobacterial species has been the center of much of this work. The intact plasmid contains 4837 base pairs with 65% G+C content and five open reading frames (ORFs). Further analysis identified the origin of replication, and that of the five ORFs, three ORFs (numbers 1, 2 and 5) are necessary for replication of pAL5000 in *M. smegmatis* (Ranes et al., 1990), but apparently only ORF 2 is required for replication in *M. fortuitum* (Villar and Benitez, 1994).

Snapper et al. (1988) developed a plasmid transformation system in mycobacteria using *M. fortuitum* / *E. coli* hybrid plasmids. The hybrid plasmid (pYUB12) contained a mycobacteria replicon from plasmid pAL5000 and an *E. coli* replicon from pIJ666. The gene for kanamycin resistance ($K^r$) was also included in the plasmid. The plasmid transformed both *E. coli* and mycobacterial species. Stable $K^r$ expression was demonstrated.

Aldovini and Young (1991) later modified this same plasmid to allow expression of other foreign proteins. They included the mycobacterial heat-shock protein, hsp60, promoter and translational start site to direct the expression of foreign genes. These regulatory elements were chosen because they are very strong promoters. Three new plasmids were produced, pY6029, pY6030 and pY6031 expressing the HIV *gag*, *pol*, and *env* genes, respectively (Aldovini and Young, 1991). These plasmids were introduced into BCG by electroporation and expression of the appropriate proteins was demonstrated by immunoblotting with HIV-positive serum. BALB/c mice were inoculated with either 5 x $10^6$ BCG-HIVgag or BCG-HIVenv intradermally or intravenously. The i.v. inoculated mice showed seroconversion rates of 3/5 to gag proteins and 1/5 to env proteins, 5 wk after immunization. None of the i.d. infected mouse sera were positive for HIV antibodies when assayed by immunoblot at this time. Levels of antibody in mice that did seroconvert were low. Cell-mediated immunity

against BCG-HIVgag as determined by IFNγ production and specific cytotoxic lymphocyte activity was also present (Aldovini and Young, 1991).

Kameoko et al. (1994) used a hybrid shuttle plasmid of pIJ666-pAL5000 (pIJK) to express an immunodominant domain of the HIV envelope V3 region, a "T-cell epitope." Having inoculated BALB/c mice s.c. with 1 $x10^6$ rBCG, they were able to demonstrate a specific CTL response two weeks after immunization.

Ranes et al. (1990), after sequencing the pAL5000 plasmid, constructed pRR3, a "mini" mycobacterial vector containing only those elements required for replication in *M. smegmatis*, BCG and *E. coli* as well as the genes for kanamycin and ampicillin resistance. This plasmid replicates in *E. coli* and was introduced into BCG and *M. smegmatis* with relatively high efficiency. The $K^r$ gene expression was confirmed by immunodetection.

The expression of HIV *nef* gene in BCG was made possible by the inclusion of the groES/groEL1 operon from *Streptomyces albus* and a synthetic ribosome binding site. Two new plasmids were constructed in this way, pWRIP17 and pWRIP19. Lymph node cells from mice immunized with BCG transformed with either of these plasmids proliferated in response to Nef protein (Winter et al., 1991).

The *lacZ* gene was inserted into plasmid pRR3 under control of the promoter $P_{AN}$ from *M. paratuberculosis* to produce the plasmid pAM320 (Lagranderie et al., 1993). BCG transformed with pAM320 produced β-galactosidase (β-gal). Guinea pigs immunized by i.d., respiratory and oral routes showed specific cellular responses to β-gal (as measured by proliferation of lymph node cells and induction of DTH) as well as β-gal-specific antibody responses. It is worth noting that guinea pigs immunized by the oral and respiratory routes displayed stronger DTH than those immunized intradermally.

Priming with non-recombinant BCG can result in potentiation of specific antibody responses and somewhat reduced T-cell responses when mice are subsequently immunized with rBCG expressing the HIV *nef* gene or the *lacZ* gene (Gheorghiu et al., 1994).

Stover and others developed another family of plasmid vectors used for transforming mycobacteria. They have identified a 1.8 Kb segment from pAL5000 (oriM), which supports plasmid replication in mycobacteria (Stover et al., 1991). They included this segment along with the *E. coli* replicon from pUC19, a gene for kanamycin resistance, and the regulatory sequences of the BCG 60 kDa heat shock protein hsp60 in the extrachromosomal plasmid vector pMV261. The regulatory sequence contains a mycobacterial promoter, a multiple cloning site including ten unique restriction sites, and a transcriptional terminator rrnABt. Using pMV261, several proteins were expressed at high levels including the *E. coli lacZ* gene, HIV *pol*, HIV *gag*, and HIV gp120. A mycobacterial genome integrating vector was also constructed by replacing the oriM sequence of pMV261 with DNA encoding the attachment site and integrase gene of mycobacteriophage L5. Expression of the protein from the integrative vector (pMV361) was less than that of the extrachromosomal vector, but increased in response to stress (Stover et al., 1991).

Using the plasmid pMV206 as a basis, various plasmids were constructed to express the pneumoccal surface protein (pspA) of *Streptococcus pneumoniae*. Modified vectors allowed expression of the pspA as a cytoplasmic product, a secreted product, or a membrane associated lipoprotein. A description of the plasmids and their construction follows (Langermann et al., 1994b).

pMV206 contains a mycobacterial origin of replication, an *E. coli* origin of replication, a kanamycin resistance gene, and a multiple cloning site. pMV261 includes everything in pMV206 as well as the promoter and ribosomal binding site of the mycobacterial hsp60. Plasmid pMV261-pspA contains the pspA antigen fused to the first

six codons of the hsp60. Since no secretion signal is included, the product is strictly cytoplasmic.

pRB26 is composed of pMV206 plus the hsp60 promoter only, allowing the inclusion of the natural pspA secretion signal peptide and ribosomal binding site in pBR26-pspA33. Thus, the expressed product is secreted. p2619 was constructed by including in pRB26 the ribosomal binding site and the 5' lipoprotein signal sequence of the *M. tuberculosis* 19kD lipoprotein (Mtb19) downstream from the hsp60 promoter. The pspA sequence is fused to Mtb19 in the plasmid p2619-pspA33. Mycobacteria transformed with this plasmid express pspA as a surface lipoprotein.

All of these plasmids exist as multicopy extrachromosomal plasmids in BCG and expression was very high (up to 15% of total BCG protein). All mice infected with BCG transformed with these plasmids produced an antibody response, however, protection from an i.p. challenge with $10^4$ *S.pneumoniae* (100 x LD50) was only observed in those animals infected with rBCG expressing pspA as a secreted product or as a surface glycoprotein (Langermann et al., 1994b).

The outer surface protein (OspA) of *Borrelia burgdorferi* was expressed using plasmids constructed in a similar way (Langermann et al., 1994a; Stover et al., 1993). In this experiment, only mice immunized with the rBCG expressing OspA as a chimeric lipoprotein developed high antibody titers and showed protection from challenge with virulent *B. burgdorferi*. Neither of the preceding studies involved an examination of the cell-mediated response.

pMV262 (identical to pMV261 but with a different reading frame) has been used to express the Leishmania surface proteinase gp63 (Connell et al., 1993; Flynn, 1994). Mice were immunized with $10^5$ BCG containing this plasmid by i.v. route or $10^6$ by the s.c. route and then challenged with $10^4$ *L. major* promastigotes. CBA/J mice generally resolve lesions spontaneously and unimmunized mice did so, but lesion size was smaller in mice immunized with the rBCG prior to infection. BALB/c mice, however, do not

spontaneously resolve their lesions and the lesions progressed even after immunization. Some protection from infection with *L. mexicana* amastigotes was shown by immunization with BCG transformed with pMV262::gp63 (Connell et al., 1993).

The pMV271 plasmid (identical to pMV261, except that it uses the hsp70 promoter instead of hsp60) has been used by Yasutomi to produce rBCG expressing the simian immunodifficiency virus (SIV) gag protein. This system was able to induce SIV-specific CTLs in rhesus monkeys (Letvin, 1993; Yasutomi, 1994).

Table 1-1 lists some of the experiments that have used recombinant BCG for immunization and the immune response elicited. In most cases, relatively high doses of rBCG have been administered. Two groups have looked at lower doses. Fuerst et al. (1991) have examined the immune response in mice immunized by the i.v. route with $10^2$ BCG expressing the *E. coli lacZ* gene. They report production of anti-β-gal-antibodies, although not as much as with higher doses, and a strong CTL response at this dose. Intranasal immunization with $10^3$ rBCG expressing *B. burgdorferi* outer surface protein A (OspA) did not develop a significant antibody titer (approx. $10^2$), but the state of CMI was not examined (Langermann et al., 1994a).

There is little data available on the effect of dose on the immune response to a foreign protein expressed in BCG. Furthermore, there are no detailed studies of the helper T cell response to such proteins. I feel that the work presented here on the immune response to a wide range of doses of rBCG, including very low doses, and an examination of how the Th1/Th2 balance induced by such vaccination depends on dose, will have a significant impact on the future work in this area.

**Table 1-1** Experiments that have examined the immune response to foreign antigens expressed in BCG.

| *Antigen* | *Animal Model* | *Expression system* | *Dose (cfu)/Route* | *Immune Response* | *Reference(s)* |
|---|---|---|---|---|---|
| HIV gag | Mouse | pIJK | ND | ND | (Matsuo et al., 1987) |
| HIV Nef | Mouse | pRR3 | $10^7$ s.c. | Proliferation | (Winter et al., 1991) |
| *E. coli* β-gal | Mouse | pMV261 | $10^6$ i.v.<br>$10^4$ i.v.<br>$10^2$ i.v. | Ab<br>Ab<br>Ab, CTL | (Fuerst et al., 1991) |
| HIV gag, pol, env | Mouse | pY60 | $5x10^6$ i.d., i.v. | IL2 & IFNγ, CTLs, antibody | (Aldovini and Young, 1991) |
| *E. coli* β-gal | Mouse | pMV261 | $10^6$ i.v.<br>$10^6$ i.p.<br>$10^4$ i.d.<br>$10^2$ i.v. | Ab, CTL, IFNγ<br>Ab<br>Ab<br>Ab, CTL, IFNγ | (Stover et al., 1991) |
| HIV gp120 | Mouse | pMV261 | $10^6$ i.v. | CTL | (Stover et al., 1991) |
| SIV gag | Rhesus Monkey | pMV271 | ? | CTL | (Yasutomi et al., 1993) |
| *E. coli* β-gal | Guinea Pig | pRR3 | $6x10^{10}$ Oral<br>$6x10^9$ Resp.<br>$10^6$ i.d. | Ab, serum TNF, T Cell Proliferation for all routes | (Lagranderie et al., 1993) |

| *Antigen* | *Animal Model* | *Expression system* | *Dose (cfu)/Route* | *Immune Response* | *Reference(s)* |
|---|---|---|---|---|---|
| SIV gag | Rhesus Monkey | pMV271 | $10^6$ | CTL | (Letvin et al., 1993) |
| *B. Burgdoferi* (OspA) | Mouse | pMV261<br>pMV251<br>p19PS | $10^6$ i.p. | Ab, Protection | (Stover et al., 1993) |
| HIV env | Mouse | pIJK | $10^6$ | CTL | (Kameoka et al., 1994) |
| *B. Burgdoferi* (OspA) | Mouse | pMV261 | $10^8$, $10^6$, $10^5$, $10^3$ intranasal. | IgA, IgG, Protection | (Langermann et al., 1994a), (Stover et al., 1994) |
| *S. pneu-moniae* (pspA) | Mouse | pMV206<br>pMV261 | $10^6$ i.p. | Ab, Protection | (Langermann et al., 1994b), (Stover et al., 1994) |
| HIV env | Guinea Pig | pIJK-V3 | $10^6$ s.c. | CTL | (Kameoka et al., 1994) |
| *E. coli* β-gal | Mouse | pRR | ? Oral<br>? Resp.<br>? i.d. | Mucosal and serum Antibody | (Gicquel, 1994) |
| Leishmania gp63 | Mouse | pMV262 | $10^5$ i.v.<br>$10^6$ s.c. | Protection<br>Protection | (Connell et al., 1993) |
| Pertussis/Tetanus toxoid | Mouse | pBMX | $5x10^6$ i.p.<br>$5x10^6$ i.v. | Serum Antibody<br>IL-2 production | (Abomoelak et al., 1999) |

Note: Column 5 lists only immune responses that were assayed. In some cases, a particular class of immunity may have been present that was not assayed for, or was not reported in the reference.

## 1.5 Research Objectives:

i. To establish a methodology for accurately enumerating ex-vivo antigen-specific cytokine-producing cells for use in characterizing the immune response of immunized mice.

ii. To characterize the BCG-specific Th cell response of mice after immunization with various doses of BCG administered by the i.d. and i.v. routes. The immune response to low doses of BCG antigen is of particular interest as low antigen doses in other systems have been shown to induce an almost exclusive Th1 response.

iii. To show that vaccination with low doses of BCG will establish a predominantly Th1 response. It is believed that such a response would provide effective protection from *M. tuberculosis* infection.

iv. To assess the hypothesis that low dose immunization will establish low zone immune deviation, by challenging low dose-immunized mice with high doses of BCG and examining the subsequent Th cell response to the challenge.

v. To determine if low dose immunization with rBCG expressing a foreign protein can establish low zone immune deviation to the expressed protein. Such deviation would be beneficial for immunizing against pathogens that are only controlled by a cell-mediated immune response.

# 2 MATERIALS AND METHODS

## 2.1 Mice

Female mice were used in all experiments. BALB/cJ mice were obtained from the animal colony at the Department of Microbiology and Immunology, University of Saskatchewan. C57BL/10 (B10) and B10.A mice were obtained from the Jackson Laboratory, Animal Resources Centre, Bar Harbor, ME, USA. All mice were housed at the animal care facility within the Department and were at least 6 wk old at the time of immunization. Animal care and treatment was in accordance with standards approved by the Canadian Council on Animal Care.

## 2.2 Growth and Enumeration of BCG

Dr. Emil Skamene of McGill University provided *M. bovis* strain BCG Montreal. The mycobacteria were propagated in Dubos medium containing 0.5% bovine serum albumin and 0.05% Tween 80. Bacteria were enumerated by plating various dilutions on Dubos agar plates after sonication for five seconds at a power output of 5 and 50% duty cycle to break up clusters of bacteria. Colonies were counted fourteen days after plating, and consequently numbers of bacteria are expressed as cfu.

## 2.3 Immunizations

For BCG immunization, 14 day cultures of BCG were used. The cells were pelleted by centrifugation at 8000 *g* for 30 min. and washed three times by repeated resupension in saline containing 0.05% Tween 80 followed by centrifugation at 8000 *g* for 20 min. After the final wash, the mycobacteria were resuspended in 1/100 of the original volume of culture

and serial dilutions were performed from this stock as needed. BCG immunizations were performed either by the i.d. route or the i.v. route. For i.d. injection, hair was removed from the abdomen using a commercial depilatory (Neet) two days prior to injection. Injections were done using a 1 cc syringe with a 30 gauge needle attached. Mice were anaesthetized with metafane inhalation anaesthetic. The skin of the abdomen was stretched over the middle finger to prevent it from bunching up in front of the needle. With the bevel of the needle facing upward, the needle was inserted several millimeters horizontally into the skin and 25-50 µl of the BCG suspension containing the appropriate number of cfu was injected. For i.v. injection, mice were first warmed under a heat-lamp to induce vasodilation. The desired number of BCG cfu were injected in a 50 µl volume into the dorsal tail vein using a 28 gauge needle.

Mice were immunized by the i.p. route with recombinant β-gal (Sigma Chemicals, St Louis, MO), either as a soluble protein in phosphate-buffered saline (PBS) or adsorbed to the aluminum hydroxide gel adjuvant, alhydrogel (Superfos Biosector, Vedbaek, Denmark) as adjuvant. For the alhydrogel preparation, the required amount of protein was mixed with alhydrogel and then left for two hours at ambient temperature to allow the protein to adsorb. An equal volume of PBS was added to the mixture and 200 µl was injected into each mouse.

Injections of purified recombinant influenza nucleoprotein as described in the section 2.20, were also performed by the i.p. route after adsorption of the protein to aluminum hydroxide gel adjuvant as described. Mice were immunized initially at six wk of age with 25 µg of purified protein and boosted an additional three times with 10 µg of protein at two week intervals, starting four weeks after the initial immunization.

In the experiment in which the IFNγ response to leishmania parasite was determined, BALB/c mice were injected with approximately 1000 *L. major* strain MHOM parasites subcutaneously into the footpad.

## 2.4 BCG Antigen Preparation

Bacteria were grown until they reached approximately $4x10^7$ cfu/ml. They were pelleted by spinning for 20 minutes at 8000 *g*, resuspended in 0.05% Tween 80 in saline, and washed twice more in this solution. The bacteria were resuspended in 5 ml of ice-cold 0.05% Tween 80 in saline, and sonicated for 14 cycles of one minute each in a Branson Sonifier, Model 450, according to the manufacturer's instructions for disrupting mycobacteria. The suspension was held in an ice bath during sonication and between cycles to prevent overheating which would destroy some antigen components. In each case, protein concentration was determined by the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rochford, Illinois) using bovine serum albumin (BSA) as a standard. The antigen was stored at -70°C. This sonicated suspension was used as antigen in the enzyme-linked immunospot (ELISPOT) assay, the bioassays, and the enzyme immunoassay (EIA). For stimulation of splenocytes in the ELISPOT assay and the bioassays, this antigen preparation was used at 3.33 μg/ml, the concentration determined to be optimal for stimulating the production of cytokines by BCG-primed spleen cells

## 2.5 SDS-PAGE and Immunoblotting

Discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Biorad Mini-Protean II electrophoresis system according to the instructions provided by the manufacturer. Gels containing 12% acrylamide (30:0.8 ratio of acrylamide to bisacrylamide) with 4% stacking gels were run in all cases under denaturing

conditions. Pre-stained molecular weight standards (Biorad or New England Biolabs, Mississauga, ON) were run on each gel.

Proteins were transferred to Biorad PVDF membranes using the Biorad Trans-Blot SD semi-dry transfer system. For immunostaining of protein, blots were first placed in a blocking solution of 1-% w/v polyvinylpyrrolidone (Aldrich Chemical, Milwaukee,WI), 0.05% Tween 20, in Tris-buffered saline (TBS) for one hour. After rinsing with TBS containing 0.05% Tween 20 (TTBS), the primary antibody was added in a solution of TBS containing 2% bovine serum albumin (BSA) and 2% Tween 20. For staining of β-gal, a monoclonal mouse anti-β-gal antibody (Sigma Chemical, St. Louis, MO) was used at a 1/1000 dilution. Staining of the His•Tag-nucleoprotein fusion was performed with an anti-6xHis monoclonal antibody (Clontech Laboratories, Inc., Palo Alto, CA) at a 1/4000 dilution. Lysates from rBCG transformed with the pMV361::NP vector were stained with serum diluted 1/100 from mice immunized with purified recombinant nucleoprotein (NP) prepared as described. After a one hour incubation and washing with TTBS, the secondary antibody, alkaline phosphatase-conjugated $F(ab')_2$ goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA), was applied at 1/2000 dilution in TBS with 2% BSA and 2% Tween 80, and incubated for 1 h. The blots were stained with NBT/BCIP color development solution (Biorad) in 0.1 M Tris-HCl, pH 9.5, containing 0.5mM $MgCl_2$.

## 2.6 Preparation of Spleen Cells for Immunoassays

Mice were killed by cervical dislocation and their spleens were removed aseptically. The spleens were placed in 10 ml of Liebovitz media and single cell suspensions of the splenocytes were prepared by passing them through a stainless steel mesh. The cells were centrifuged and resuspended in complete RPMI medium (RPMI 1640 supplemented with L-glutamine, containing 10% heat-inactivated fetal bovine serum

(Gibco-BRL), penicillin (100 U/ml) and streptomycin (100 μg/ml) and $5x10^{-5}$ M β-mercaptoethanol). Viable leukocytes were counted using trypan blue exclusion and a haemacytometer. Cells were kept on ice until they were aliquoted for the ELISPOT assays or bioassays.

## 2.7 ELISPOT Assay for Detection and Enumeration of Antigen-Dependent IFNγ- and IL-4-Secreting Cells

96-well nitrocellulose-bottomed plates (Polyfiltronics, Rockland. Mass) were coated with purified anti-IFNγ antibody (R4-6A2) or anti-IL-4 antibody (11B11) (Pharmingen, San Diego, Calif.) by incubating the plates overnight at 4°C with 100 μl/well of antibody at 1.25 μg/ml in 50 mM carbonate buffer, pH 9.6. Spots appeared to be of highest quality when the coating buffer was between pH 9 and 10. The plates then were emptied and rinsed once with 200 μl of complete RPMI medium. To reduce non-specific binding of proteins at later stages in the assay, the plates were incubated for at least one and up to three hours with an additional 200 μl of complete RPMI, which was discarded immediately prior to the addition of spleen cells.

The spleen cells from experimental mice were added to the wells at densities ranging from $1.25x10^5$ to $1x10^6$ splenic leukocytes per well. When required, additional spleen cells from unimmunized mice were added to wells in an additional 100 μl of medium to bring the number of splenic leukocytes up to a total of approximately $10^6$/well. Wells without additional feeder cells received an additional 100 μl of complete RPMI. The antigen, when required, was added to the wells with the feeder cells or additional medium as described above. For stimulation of cells in the ELISPOT, BCG antigen was

used at a concentration of 0.66 μg/well, leishmania antigen (Power et al., 1999) was used at a concentration of 3 μg/well, and soluble β-gal was used at 1 μg/well. The assay was performed in triplicate wells with and without antigen stimulation for each cell density in all experiments. The seeded plates were placed undisturbed in a 37°C incubator for 8 h. I have compared the ELISPOT results after incubation of cells in the ELISPOT plates for 8, 16 and 24 h. There was no significant difference between the number of antigen-specific IFNγ-spots generated by spleen cells at these times. However, the longer incubation times resulted in an increase of non-specific spots in wells containing no antigen (unpublished data). After incubation, the plates were rinsed once with distilled water and washed thoroughly with PBS containing 0.05% Tween 20 (PBST). Biotinylated anti-IFNγ (XMG1.2) or anti-IL-4 (BVD6-24G2) antibodies, (Pharmingen) at 1.25 μg/ml in PBST were added to plates at 100 μl/well and incubated at 4°C overnight. The plates were washed again with PBST and 100 μl of alkaline-phosphatase strepavidin (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.) at a concentration of 0.2 μg/ml in PBST was added to each well and incubated at room temperature for 1.5 h. The plates were washed by repeated submersion in reverse osmosed deionized water ($rdH_2O$). Spots were developed by the addition of 100 μl/well of NBT/BCIP substrate (Boehringer-Mannheim, Germany) in substrate buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 0.05 M $MgCl_2$) prepared according to the manufacturer's instructions. After spot development, the plates were emptied, rinsed thoroughly with $rdH_2O$ and allowed to dry. Spots were counted with the aid of a dissecting microscope. Only the number of antigen-dependent cytokine-producing cells is reported in each case. This number is assessed by determining the number of spots in wells that have been stimulated with

antigen and subtracting the number of spots obtained from wells in the absence of antigen. In all ELISPOT assays, spleen cells from unimmunized mice were run to ensure that spot formation in the assay required previous immunization with antigen.

I use the term *feeder cells* to refer to spleen cells from unimmunized mice which are added to wells containing spleen cells from antigen primed mice to increase the overall cell density in the well. These feeder cells are required to achieve optimum sensitivity in the assay if the immune cells are seeded at densities lower than $10^6$ cells/well. For the experiments in which BALB/c mice were used, the feeder cells consisted of splenic leukocytes from syngeneic non-antigen-primed mice prepared in the same manner as the spleen cells from the immunized mice. For the experiments in which the B10 and the MHC-congenic B10.A mice were used, the feeder cells consisted of either B10 or B10.A spleen cells, which were prepared as above and further depleted of T cells by incubation with anti-Thy-1 antibody and complement. This was done to eliminate the possibility of the feeder cells responding to the allogeneic MHC-molecules of the immune cells.

The ELISPOT was also performed on Thy-1$^+$-depleted immune cells to ensure that the IFNγ spots were indeed produced by T cells. In some cases, the assay was performed on CD4$^+$- and CD8$^+$-depleted cells and the results were compared to results obtained with undepleted cells to determine the phenotype of the cytokine-producing cells. The T cell depletions were carried out as indicated below.

### 2.8 Complement-Mediated Depletion of Thy-1$^+$, CD4$^+$, and CD8$^+$ Cells

Spleen cells were suspended at $10^7$/ml in complete RPMI medium. Ascitic fluid containing the anti-Thy-1.2 (TIB 99), anti-CD4 (GK1.5), or anti-CD8 (TIB99) antibodies (ATCC, Rockville Maryland), was added to the cell suspension at a dilution of 1/200 and the cells were incubated on ice. After 1.5 h, rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added at 1/10 the volume of the cells and the mixture was

incubated at 37°C for 45 min. The cells were washed twice with complete RPMI medium and resuspended in fresh medium. The number of viable white cells was counted once again to check the percentage of lysis. In procedures where complement mediated lysis of cells was performed, cells were added to the assay based on the cell counts obtained before the depletion.

## 2.9 Interferon-γ Assay

### 2.9.1 Preparation of Cellular Supernatant Fluid

Spleen cells from BCG-infected and control mice were plated at a concentration of $4x10^6$ cells/well in 24 well trays in complete RPMI medium. Cells from each mouse were incubated both with antigen and without antigen to ensure that cytokine production was antigen-dependent. When present, antigen was added to the wells at a concentration of 3.3μg/ml. After incubation at 37°C for 48 h, the supernatant fluid was collected. Cells and cellular debris were removed by centrifuging at 500 *g* for 20 minutes. The samples were stored at -20°C until use.

### 2.9.2 Interferon-γ Bioassay

IFNγ present in spleen cell culture supernatants was quantitated using a viral cytopathic reduction assay employing the murine fibroblastic cell line L929 and endomyocarditis virus (Familletti et al., 1981). The reduction in viral cytopathogenicity was invariably due to interferon as confirmed by its abrogation upon adding the IFNγ neutralizing monoclonal antibody XMG1.2 (Cherwinski et al., 1987). The assay was standardized employing recombinant murine IFNγ of known activity, as specified by the manufacturer (Intermedico, Markham, ON). All assays were done in triplicate. The

production of IFNγ is given as units per $10^6$ cultured white spleen cells. One ng of IFNγ is equal to 5 units according to the standard assay procedure.

## 2.10 Analysis of Anti-Mycobacterial or Anti-β-gal Antibody by Enzyme Immunoassay

Blood from normal or infected mice was collected by tail bleeding into serum separator tubes (Becton Dickinson, Rutherford, NJ). After allowing the samples to clot for at least several hours, individual serum samples were harvested by centrifugation at approximately 5000 *g* for 60 seconds. Sera were stored at -70°C until use. The IgG1 and IgG2a serum antibody titers were determined using an EIA (Hornbeck, 1994). Immulon-4 96 well polystyrene plates (Dynatech Laboratories, Chantilly, VA) were coated with either BCG antigen or with soluble β-gal antigen at a concentration of 1 μg/well in 100 μl of PBS. Serum samples were diluted 1/100 in PBS containing 2% BSA and 2% Tween-20 (assay diluent) and two-fold serial dilutions were prepared in the EIA plate. The plates were incubated at 37°C for 2 hr and washed thoroughly with $rdH_2O$. Horse-radish peroxidase-labeled rat anti-mouse monoclonal antibody against mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was added to the wells at a dilution of 1/3000 in assay diluent, 100 μl per well, and incubated at 37°C for two hours. After washing, ABTS (2.2'-azino-di[3-ethyl-benzthiazoline sulphonate (6)]) substrate solution (Kirkegaard and Perry, Gaithersburg, MA) was added to the wells and incubated for 20 minutes before reading the plates on a Biorad (Hercules, CA) Model 2550 EIA Reader. Pooled serum from normal mice was used as a control. Titers were determined in one of two ways. For the results presented in Chapter 4, the titer is presented as the last dilution factor to give an optical density in the assay twice the value of the result for a pool of sera from unimmunized control mice. At the suggestion of my supervisory committee,

I used a different method to determine titer in subsequent experiments. For results presented in Chapter 5, optical density was plotted against the log of the dilution factor for each sample. The endpoint titer was assigned as the point on the line at which the optical density was 0.5 above the value for wells in which no serum was added (conjugate blank).

### 2.11 Establishment and Assessment of Cell-Mediated Immune Deviation

Mice were inoculated intravenously in the tail vein or intradermally on the abdomen with the number of cfu indicated in the text, and were later challenged with a high number of BCG cfu given intradermally. The number of mycobacterial antigen-specific IFNγ- and IL-4-producing cells in the spleen of these high dose challenged mice was assessed by the ELISPOT assay. The number of IFNγ-producing cells and number of IL-4-producing cells were taken as measures of the Th1- and Th2-components of the immune response respectively.

### 2.12 Isolation of plasmid DNA from *E. coli*

Plasmid isolation was carried out using two methods. In most cases, plasmid DNA minipreps were prepared by the boiling method (Sambrook et al., 1989). In some cases, when DNA of higher purity was required, DNA midipreps were prepared using a commercial kit (Qiagen, Mississauga, ON,). In the latter case, the concentration was estimated spectrophotometrically by absorbance at 280 nm wavelength.

### 2.13 Mycobacterial Expression Vectors

Dr. Mark Hanson of MedImmune, Gaithersberg, MA, kindly provided two mycobacterial expression vectors, pMV261 and pMV361. Both plasmids contain the *E.*

*coli* replicon from pUC19 which supports replication in *E. coli*, the kanamycin resistance gene, and the regulatory sequences of the BCG heat shock protein hsp60. The regulatory sequence contains a mycobacterial promoter, a multiple cloning site including ten unique restriction sites, and a transcriptional terminator rrnABt. The extrachromosomal plasmid vector pMV261 (Figure 2-1) contains a 1.8 kb segment of DNA from pAL5000, a natural plasmid obtained from *M. fortuitum*, which includes a mycobacterial origin of replication (oriM) to support plasmid replication in mycobacteria. pMV361 (Figure 2-2) is an integrative vector constructed by replacing the oriM sequence of pMV261 with DNA encoding the attachment site and integrase gene of mycobacteriophage L5. In both cases, inserted genes are expressed as a fusion protein with the first six amino acids of the mycobacterial hsp60. Vectors pMV261 and pMV361 containing genes for β-gal (pMV361::*LacZ*) and the gag protein of HIV (pMV361::Hgag) were also provided by MedImmune.

## 2.14 Growth and Purification of Influenza Virus

Thirty-five 10-day old chicken embryos were inoculated in the allantoic sac with 100 µl of influenza A/PR/8/34 virus stock (local laboratory stock, hemagglutination titer of 1/256) according to the method of Rovozzo and Burke (1973). The eggs were incubated for 3 days at 37° C, and turned each day. On the third day, the embryonated eggs were placed in the refrigerator overnight. The virus-containing allantoic fluid was collected, pooled and centrifuged at 1000 *g* for 10 min to remove particulate matter. The titer of the subsequent virus stock was 1/256 as assessed by virus hemagglutination of chicken red blood cells. The virus was further purified by the method of Laver (1969).

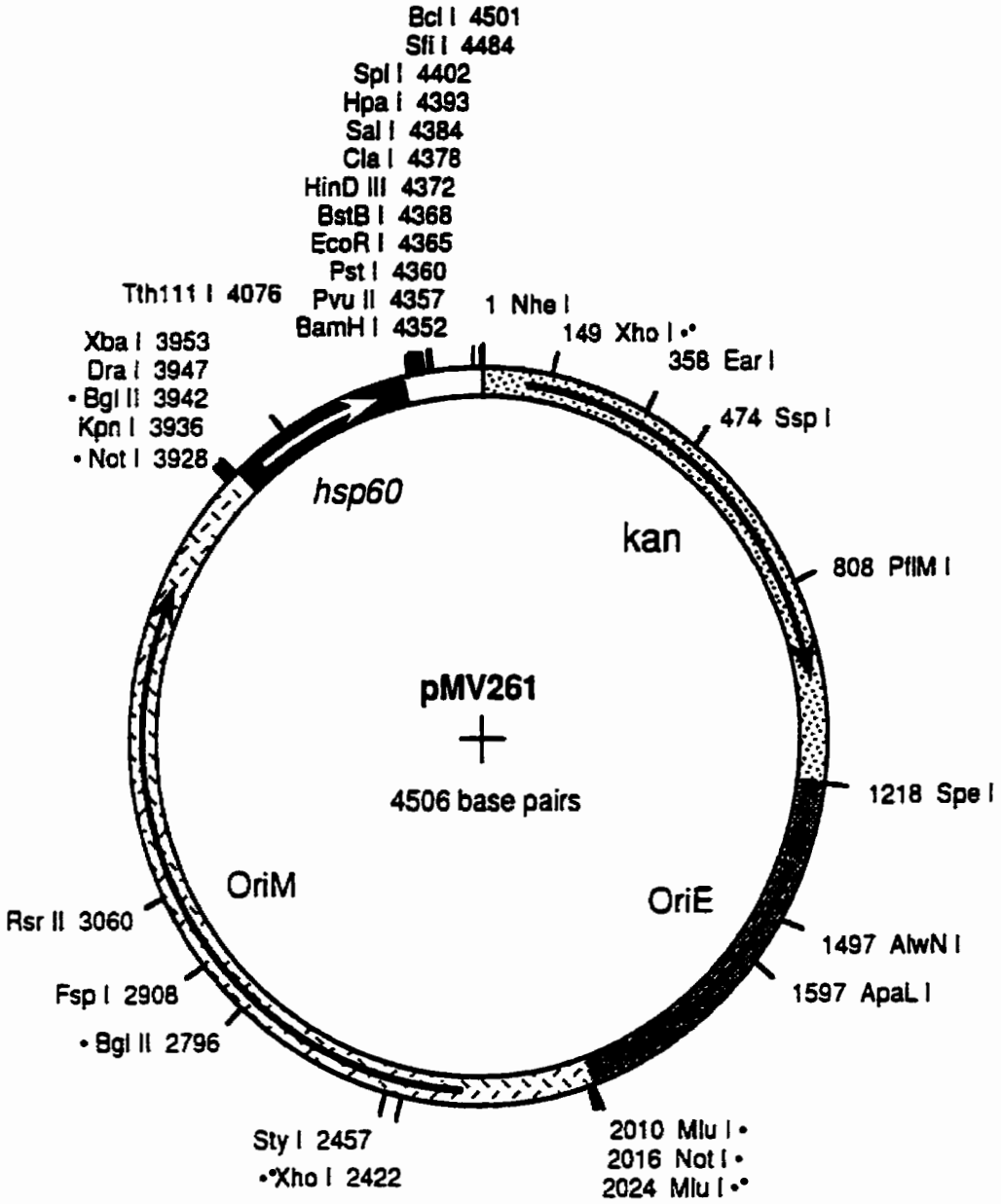



**Figure 2-1** Structure of plasmid pMV261.

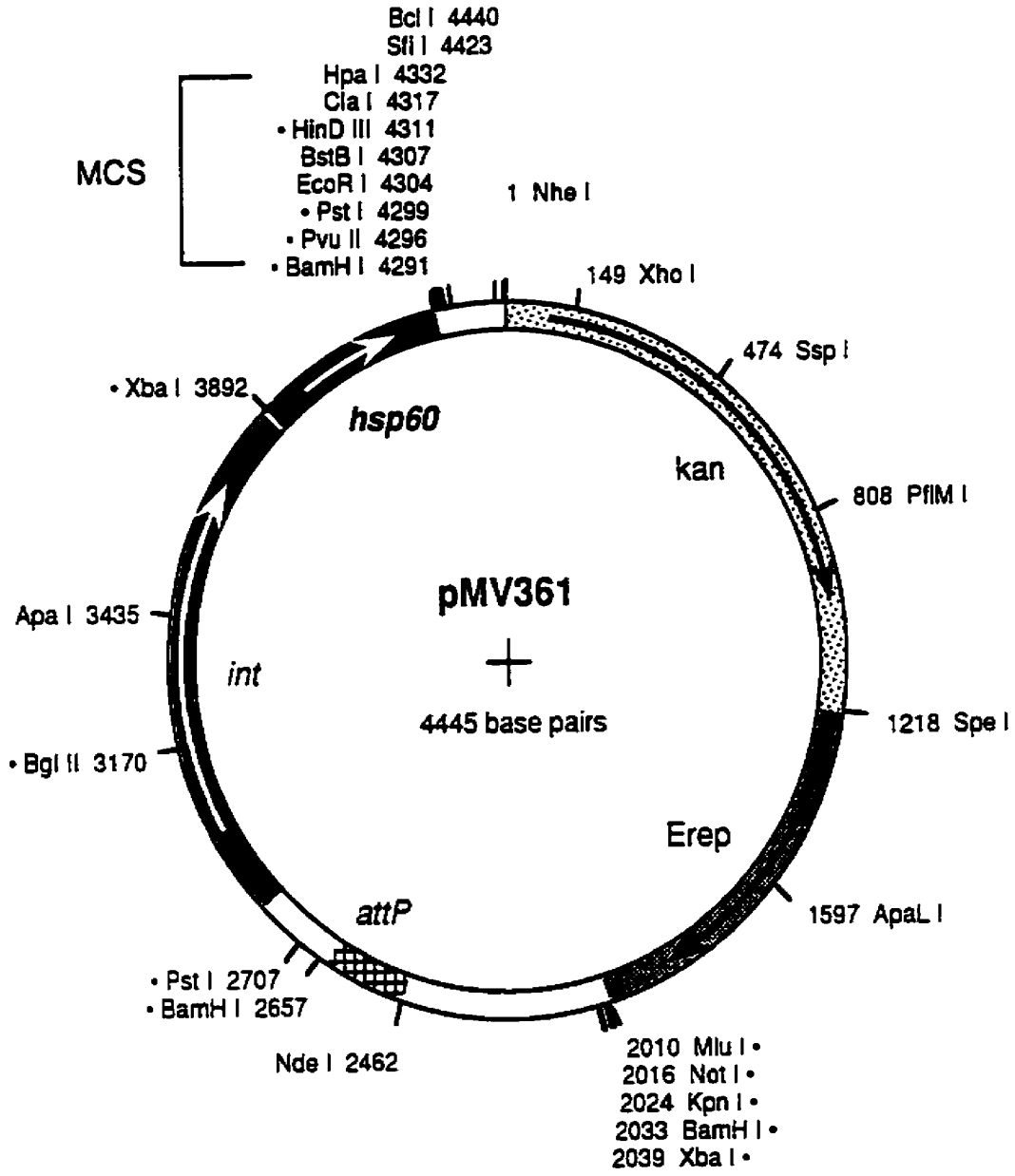



**Figure 2-2** Structure of plasmid pMV361.

The virus infected allantoic fluid was centrifuged at 2000 *g* for 15 min to remove particulate matter. The supernatant fluid was chilled to 0°C in an ice-water bath. Chicken blood cells were collected in Elsevers solution as anti-coagulant. The cells were washed in PBS and also chilled in the ice bath. A 5% v/v of packed red blood cells was added to the allantoic fluid and the mixture was stirred gently for one hour while kept in the ice-bath. The supernatant fluid was then removed and discarded. The agglutinated cells were washed once with ice-cold saline, and then resuspended in a volume of warm saline equal to 1/10 of the original volume of allantoic fluid. The virus was then allowed to elute for one hour at 37°C. The cells were removed by centrifugation and the supernatant fluid containing the virus was collected and stored at -70°C.

### 2.15 Isolation of Viral RNA

The purified virus was pelleted by centifugation at 100,000 *g* for 16 h. The viral RNA was extracted by the following method. The virus pellet was suspended in 0.5 ml of RNA buffer (0.5 M NaCl, 0.2 M Tris-HCl, pH 7.6, 10 mM ethylenediaminetetraacetic acid (EDTA), and 1% SDS. A 0.5 ml volume of phenol:chloroform:isoamyl alcohol at a ratio of 25:24:1, which had been equilibrated with RNA buffer (without SDS), was added. The mixture was vortexed at high speed for 1 min and then centrifuged for 30 sec. The aqueous phase was removed and treated with an equal volume of the phenol:chloroform mixture and centrifuged at high speed to separate the phases. The aqueous phase was transferred to a clean tube and 1 ml of ice-cold 100% ethanol was added to this tube, which was then spun at high speed for 1 min to pellet the RNA. The pellet was rinsed once with 150 µl of 70% ethanol, resuspended in 100 µl of $rdH_20$, and stored at -70°C. All $rdH_20$ used in this extraction method was treated with diethylpyrocarbonate and autoclaved before use.

## 2.16 Preparation of Viral cDNA

First strand cDNA was prepared using the Superscript kit (Gibco Scientific, Burlington, ON) according to the instructions provided. Primer PR8-1 (ACG AAA AGC AGG) prepared by Integrated DNA Technologies (Coralville, IA, USA) was used for first strand cDNA preparation. This procedure provides cDNA of all eight influenza gene segments as the primer is complimentary to a sequence common to all segments.

## 2.17 Amplification of the Nucleoprotein Gene by Polymerase Chain Reaction (PCR)

To amplify the nucleoprotein gene segment, two NP specific primers were used, NP-1 (5'-CGA ATT CAT GGC GTC CCA AGG CAC C-3') and NP-2 (5'-CCA TCG ATT AAT TGT CGT ACT CCT CTG-3'). To facilitate cloning of the PCR product into the various vectors, the primers contained 5' extensions coding for restriction enzyme sites: *Eco* RI in NP-1 and *Cla* I in NP-2. As the primers anneal to templates without these extensions at a lower temperature than to those with extensions, the PCR was carried out in two phases. The denaturing time and temperature were 94°C for one min and the extension conditions were 72°c for 2 minutes throughout the procedure. In the first three rounds of amplification, the annealing conditions were 44°C for one min. The temperature was then ramped from 44°C to 72°C at a rate of 0.5°C per min. followed by the one minute extension at 72°C. Subsequently, the annealing temperature was raised to 53°C for another 30 rounds of amplification.

The NP gene segment was cloned into the *Eco* RI and *Cla I* sites of pBluescript SKII vector (Invitrogen, San Diego, CA). The construct was used to transform DH5α *E.*

*coli* cell line and miniprep DNA of transformed colonies was prepared. Sequencing was carried out using the T7 DNA sequencing kit (Pharmacia, Piscatawy, NJ). The sequence of the cloned nucleoprotein gene was compared to the original published sequence for this strain of virus (Winter and Fields, 1981).

## 2.18 Cloning of the Nucleoprotein Gene into *E. coli* Expression Vector pTrcHis

The NP gene was cloned into pTrcHis (Invitrogen Corp, San Diego, CA). For the purpose of cloning into this plasmid, the pBluescipt construct containing the NP gene was digested with the restriction enzyme *Xho I* which lies downstream from the *Cla I* site at the 3' end of the NP gene, and a *Xho* I-*Eco* RI linker was ligated into this site. This provided a NP gene insert that has an *Eco* RI restriction site at either end of the gene to facilitate cloning into vectors containing a single *Eco* RI site within the multiple cloning site. This fragment was then ligated into the *Eco* RI site of pTrcHis for expression of the NP gene. After transformation of DH5α cells, miniprep DNA prepared from individual colonies were screened for appropriately sized plasmids. Those containing the fragment in the appropriate orientation were selected based on fragment size after restriction digest.

## 2.19 Transformation of *E. coli*

Transformation of *E. coli* was performed using the *E. coli* pulser electroporator (BioRad) according to the manufacturer's instructions.

## 2.20 Expression and Purification of Recombinant NP from *E. coli*

The pTrcHis plasmid contains a His•Tag, a sequence of six histidine residues in sequence, immediately downstream from the plasmid encoded ATG start site. The NP gene was inserted in frame with this sequence so that the expressed protein would be a fusion protein with the His•Tag. The His•Tag has a very high affinity for the nickel ion which allows purification of the fusion protein from the cell lysate with an affinity media. Purification was carried out using the Novagen His•Bind metal chelation resin columns (Novagen, Madison, WI) using the directions for denaturing conditions (Novagen, 1995). Purity was assessed by SDS-PAGE.

## 2.21 Cloning of the NP Gene into the Mycobacterial Expression Vectors pMV261 and pMV361

The NP gene was cut out of pBluescript SKII by restriction digest with *Eco* RI and *Cla* I and ligated into pMV361 and pMV261 plasmids digested with these same enzymes. The ligation reaction product was used to transform *E. coli* strain DH5α. Transformed cells were plated on Lennox broth agar (US Biologicals, Swampscott, MA) plates containing 50 μg/ml of kanamycin as a selective marker. Miniprep DNA was prepared from cultures of selected colonies and screened for the appropriate plasmid size. DNA sequencing of the plasmid/insert junction was performed to ensure that the insert was in frame with the plasmid encoded portion (first six amino acids of hsp60) of the fusion protein gene.

## 2.22 Preparation of Electroporation-Competent BCG

The method of Parish and Stoker (1995) was used to prepare electroporation-competent BCG. A 200 ml aliquot of Middlebrook 7H9 medium (Difco, Detroit, MI) containing OADC supplement (Difco, Detroit, MI) and 0.05% Tween 80 was inoculated with 1 ml of a 5 day culture of *M. bovis* and cultured for 12 days. Glycine was added to the culture to a final concentration of 1.5% for the last two days of culture. At the end of the culture period, the mycobacteria were incubated on ice for 1.5 hr before harvesting. They were pelleted by centrifugation at 8000 *g* for 30 min at 4°C. The resulting pellet was washed by repeated suspension and centrifugation (8000 *g*, 4°C) in reducing volumes of an ice cold solution of 10% glycerol, first in 100 ml, then in 25 ml and lastly in 5 ml. After the final centrifugation, the bacteria were suspended in 2 ml of ice cold glycerol, dispensed into 100 µl aliquots in eppendorf tubes, and flash frozen by immersion in an ethanol bath containing dry ice. The tubes were stored at -70°C.

## 2.23 Transformation of Competent *M. bovis* BCG

Transformation of competent BCG was performed by electroporation using the Gene Pulser II (BioRad). BCG were removed from the freezer and immediately placed on ice. One microgram of plasmid midiprep DNA was added to the tube and the contents were mixed and placed on ice for ten min. The mixture was transferred to a chilled, 2 mm gap electroporation cuvette (VWR scientific, Toronto, ON). The bacteria were subjected to a pulse of 3.5 kV, 25 µF, at a resistance of 1000 Ω (Parish and Stoker, 1995). The cuvette was incubated on ice for ten minutes before the contents were transferred to a separate tube and diluted with 3 ml of Middlebrook 7H9 media (Difco, Detroit, MI) with 0.5% Tween 80 and OADC supplement (Difco). The mixture was incubated at 37°C for 3

h to allow for antibiotic expression. Cells were harvested by centrifugation at 3000 *g* for ten min and plated on Middlebrook 7H10 agar plates with OADC and 10 µg/ml of kanamycin (Parish and Stoker, 1995). The plates were sealed with parafilm and incubated at 37°C for 21-28 days to allow colonies to grow.

# 3 A Valid ELISPOT Assay for Enumeration of Antigen-Dependent Cytokine-producing Cells

## 3.1 Introduction

The ELISPOT assay for the enumeration of cytokine-producing cells was first described over a decade ago (Czerkinsky et al., 1988). Since that time, various modifications and applications of this technique have been described. Many reports on conditions for optimization of the ELISPOT assay have used T cell clones, (Czerkinsky et al., 1988; Hutchings et al., 1989; Miyahira et al., 1995) or mitogen- (Okamoto et al., 1998; Taguchi et al., 1990), or anti-CD3- (Favre et al., 1997; Hutchings et al., 1989) stimulated cells as a source of cytokine-producing cells. T cell clones or mitogen-stimulated cells are convenient for designing ELISPOT assays because they provide an efficient and consistent source of cytokine-producing cells. These investigations have defined certain parameters of the assay, such as the identification of appropriate anti-cytokine antibody pairs and the standardization of reagents. However, these experimental models do not necessarily optimize the conditions for detecting antigen-specific cytokine-producing lymphocytes present in an immune individual. The requirements for triggering antigen-specific cytokine production by a population of polyclonal lymphocytes in vitro may be different from those causing cytokine release from cells belonging to a T cell clone. Anti-CD3 and some forms of mitogen stimulation of T cells do not require antigen presentation and, therefore, are not good models for antigen-specific cytokine production. To obtain an optimally sensitive assay for detecting single, antigen-specific cytokine-producing cells, the effects of varying parameters such as the antigen concentration, the numbers and types of cells in the ELISPOT well and the time required for stimulation must be assessed.

The enumeration of antigen-dependent cytokine-producing cells in freshly isolated lymphocytes by ELISPOT assay has been problematic. Many report a requirement for a period of pre-incubation of the lymphocytes with antigen, from overnight to 72 hours or more (Hutchings et al., 1989; Xu et al., 1991; Muller et al., 1994; Sarawar and Doherty, 1994; Milligan and Bernstein, 1995), to optimize detection of cytokine-producing cells. Although very short-term restimulation with antigen may be acceptable, the activity of cells after longer term incubation with antigen in vitro may not reflect the ex-vivo state of the cells, the assessment of which is usually the primary purpose of the assay. The addition of extraneous cytokines during a pre-incubation step is inadvisable, as this will compound the difficulties of relating the observations to the in vivo state of the cells.

If the goal is to measure the magnitude of the immune response in an individual by estimating the number of antigen-dependent cytokine-producing cells in a particular immune compartment, it is necessary to make such an estimate as soon as possible after removing the cells from the individual, without long incubation periods or pre-exposure to extraneous cytokines. I describe a short-term ELISPOT assay technique for detection of antigen-specific IFNγ-producing murine splenic T cells. Although the results presented in this chapter concentrate on IFNγ-producing T cells, I have used similar experiments to show the validity of this method for enumerating antigen-specific IL-4-producing T cells. This method differs from most described in that the cells are placed directly ex-vivo into the ELISPOT plate without the need for a separate step involving pre-incubation with antigen. Optimal sensitivity is obtained in this assay by incubating the cells at high densities in the ELISPOT tray wells. The results presented here have shown that very high cell densities are required to provide effective antigen-presenting cell- (APC) function to the T cells. Therefore, when lower numbers of immune cells are used, spleen cells from normal syngeneic mice can be used to provide this APC function.

## 3.2 Results

In collaboration with others in Dr. Bretscher's lab, I have developed a very sensitive ELISPOT technique for detection of antigen-specific T cells which is performed on cells immediately after their removal from the mouse without the requirement for a separate step involving the incubation of the cells with the antigen. Sensitivity of the assay is dependent upon a high density of cells in the wells of the ELISPOT plate while they are being stimulated with antigen. In an optimally functional and valid assay, I expect that the number of cytokine-producing cells detected (spots) to be proportional to the number of immune cells in the well. Therefore, for each two-fold dilution of immune cells in the well, the number of spots should decrease by half. For the ideal assay, a plot of number of immune cells vs. the number of spots should result in a straight line that intersects the y axis at the origin. Figures 3-1 to 3-3 demonstrate the requirement for high cell density in the assay regardless of the antigen used. In the experiments shown in Figures 3-1 and 3-2, the assay was performed with two-fold serial dilutions of immune cells from mice that received approximately $10^7$ BCG cfu by the intradermal route, ten weeks earlier. When the serially diluted immune cells alone were incubated with antigen in the ELISPOT plate wells, the resulting number of spots at the lower dilutions was not proportional to the number of immune spleen cells added to the well. However, when the same assay was performed with the addition of feeder cells to bring the total number of cells in each well to approximately $10^6$, the number of spots was approximately proportional to the number of immune cells plated at all dilutions.

Figure 3-3 demonstrates a similar result using another antigen system, the leishmania parasite. The results of the ELISPOT assay for spleen cells from two individual mice infected with leishmania parasites are shown. When dilutions of these cells are used in the ELISPOT alone, the resulting number of spots is not relative to the number of cells, i.e., a plot of spots vs. the number of immune cells is not linear.

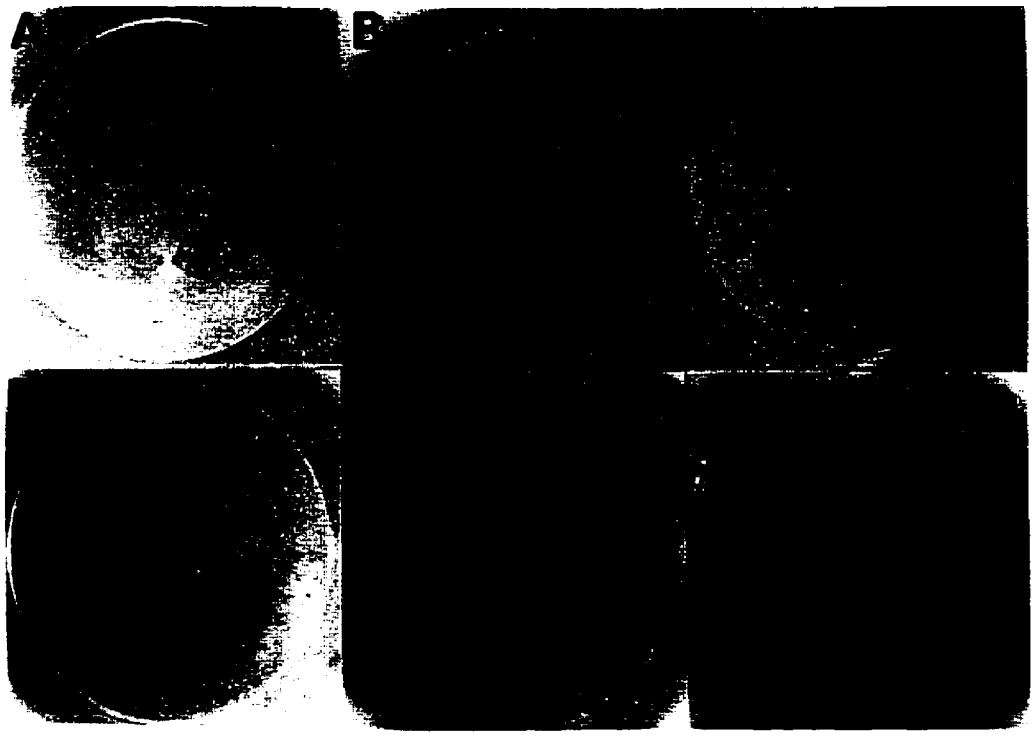


**Figure 3-1** Representative wells from the ELISPOT assay performed with and without added feeder cells. The ELISPOT technique was performed as indicated in the Materials and Methods section on serial dilutions of spleen cells from BCG immunized BALB/c mice. The wells contained $5x10^5$ (A and D), $2.5x10^5$ (B and E), and $1.25x10^5$ (C and F) immune spleen cells/well. The wells shown in A, B and C contained immune cells only whereas syngeneic spleen cells from unimmunized mice were added to the wells depicted in D, E and F to bring the total number of spleen cells/well to approx. $10^6$.

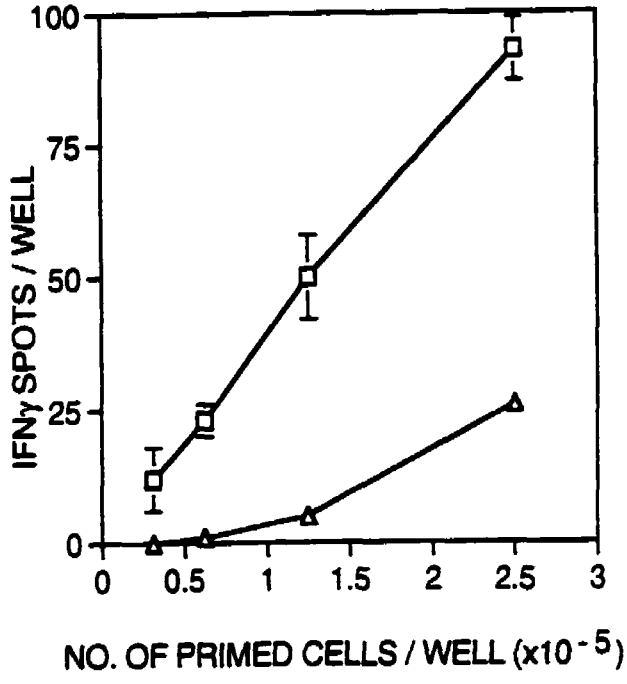



**Figure 3-2** Effect of feeder cells on the sensitivity of the IFNγ ELISPOT assay performed on spleen cells from BCG immunized mice. Mice were injected by the intradermal route with approximately $10^7$ BCG cfu. The ELISPOT assay was performed on serial two-fold dilutions of splenocytes pooled from 3 BCG immunized mice. The immune cells were incubated in the ELISPOT plate either alone (Δ) or with spleen cells from syngeneic unimmunized mice (□) to bring the total number of cells/well to $10^6$. The number of antigen-specific spots is determined by subtracting the number of spots (in all cases < 10 spots/$10^5$ spleen cells) in wells without added antigen. Each point represents the mean number of antigen-specific spots in 3 assay wells +/- SD.

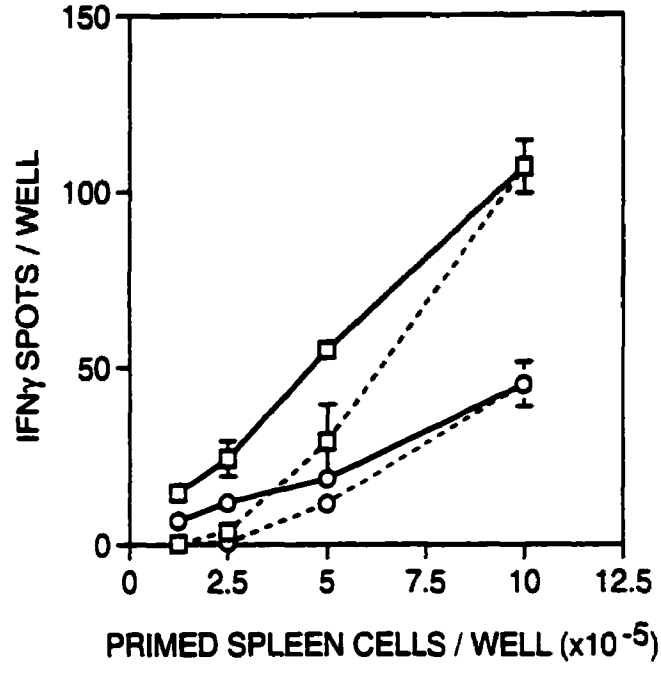



**Figure 3-3** Effect of feeder cells on the sensitivity of the IFNγ ELISPOT assay performed on spleen cells from *L. major*-immunized mice. The ELISPOT assay was performed separately on serial two-fold dilutions of splenocytes from two *L. major*-immunized mice (□,○) as indicated. The immune cells were incubated in the ELISPOT plate for 8 hours either alone (dashed line) or with spleen cells from unimmunized syngeneic mice to bring the total number of cells/well to $10^6$ (solid line). Each point represents the mean number of spots in 3 assay wells after subtracting background spots in wells without antigen (in all cases < 10 spots/$10^6$ spleen cells) +/- standard deviation (SD).

However, with the addition of feeder cells, the resulting number of spots is always proportional to the number of plated immune cells, i.e., the results are linear. The same conclusion has been made by N. Ismail with spleen cells from mice immunized with red blood cells (Power et al., 1999). In preliminary experiments, $10^6$ cells/well was shown to be optimum for spot detection in the BCG and leishmania antigen systems, as higher densities resulted in increased background without significant increases in sensitivity (unpublished data). It should be noted that the feeder cells do not significantly contribute to the number of spots produced, as $10^6$ feeder cells/well alone produce negligible numbers of spots when stimulated with antigen. These results indicate that the ELISPOT assay as described, is an efficient method to detect cytokine responses to a variety of antigens and that the requirement for high cell density to achieve maximum sensitivity in the ELISPOT assay is independent of the antigen used.

In ELISPOT assays for IL-4-producing cells, sensitivity was much less dependent on the density of cells in the well. Although increased cell density increased detection of IL-4-producing cells marginally, the difference was much less dramatic than that in the IFNγ assay.

I hypothesized that high cell density is necessary to allow T cell - T cell interactions to occur, to condition the media by providing some soluble factor required for optimal function of the T cells, or to provide adequate antigen presentation to the immune T cells. The experiments shown in Figure 3-4 were conducted to address this question of feeder cell function. In three separate experiments, I performed the ELISPOT assay on immune cells from BCG-immunized mice. In each case, immune spleen cells from these mice were incubated either alone, or with T cell-depleted feeder cells from syngeneic mice or from MHC-congenic mice. In all cases, MHC-matched feeder cells were required to ensure optimal functioning of the assay. All spots were produced by antigen-specific T cells, as spleen cells from unimmunized mice did not produce significant spots under the

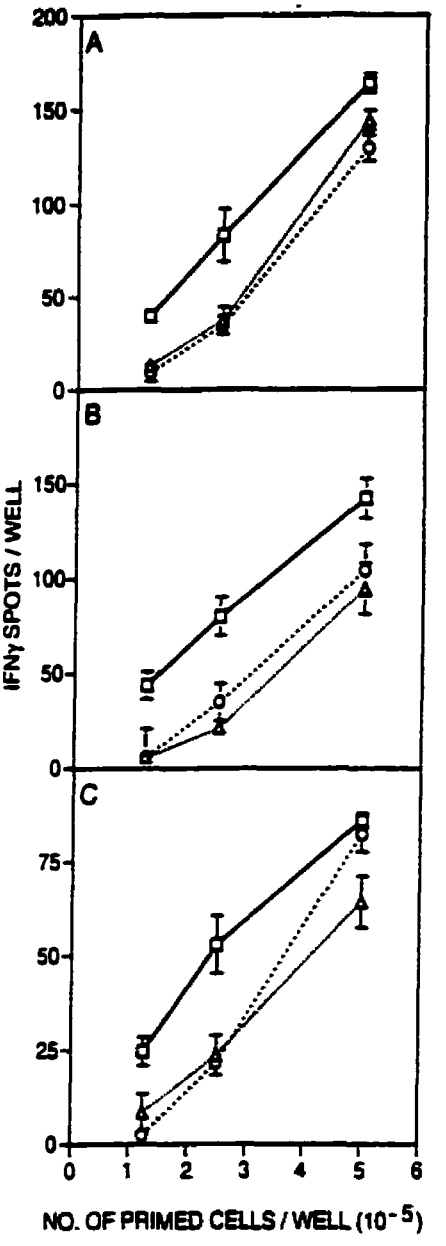



**Figure 3-4** MHC-matched feeder cells are required for enhanced sensitivity observed at high cell density. Serial dilutions of white splenocytes from BCG-immunized mice were used in the ELISPOT assay. These immune cells were incubated in the ELISPOT plate wells either alone (Δ), or with T cell depleted feeder cells from syngeneic (□) or MHC-congenic (○) unimmunized mice. A and B represent two independent experiments in which the immune spleen cells were from B10 mice ($H\text{-}2^b$) and the MHC-congenic feeder cells were from B10.A mice ($H\text{-}2^a$). C shows the reverse experiment in which the immune cells were from B10.A mice and the feeder cells were taken from B10 mice. All points represent the mean number of spots in 3 assay wells +/- SD. T lymphocyte depletion of immune cells eliminated spot formation.

same conditions and T cell depletion of spleen cells from immunized mice eliminated spot formation (unpublished data). These observations show that the major function of the feeder cells is MHC-restricted. I infer that the increase in spot formation is due to an increase in the number of antigen-presenting cells in immediate contact with the T cells, providing improved antigen presentation and thereby enhanced stimulation of the T cells.

Complement depletions performed with anti-$CD4^-$ and anti-$CD8^-$ antibodies demonstrated that in BCG-immunized mice, approximately 75% of IFNγ-producing cells were $CD4^-$ while 25% were of the $CD8^-$ phenotype. BCG-specific IL-4-producing cells were of the $CD4^-$ phenotype.

I also compared the IFNγ ELISPOT assay to the bioassay for antigen-dependent production of IFNγ that is used in Dr. Bretscher's laboratory. The results are shown in Figure 3-5. There is a high correlation between the two assays. However, the ELISPOT assay is a more sensitive assay as IFNγ spot-forming cells can be detected when IFNγ was undetectable by the bioassay. This may simply be due to differences in the sensitivity of detection between the two methods. Alternatively, it may be a result of the long term culture system used in the IFNγ bioassay. During this culture period, the cells are actively metabolizing so some of the IFNγ that is produced may be either consumed by cells, or broken down by proteases in the medium.

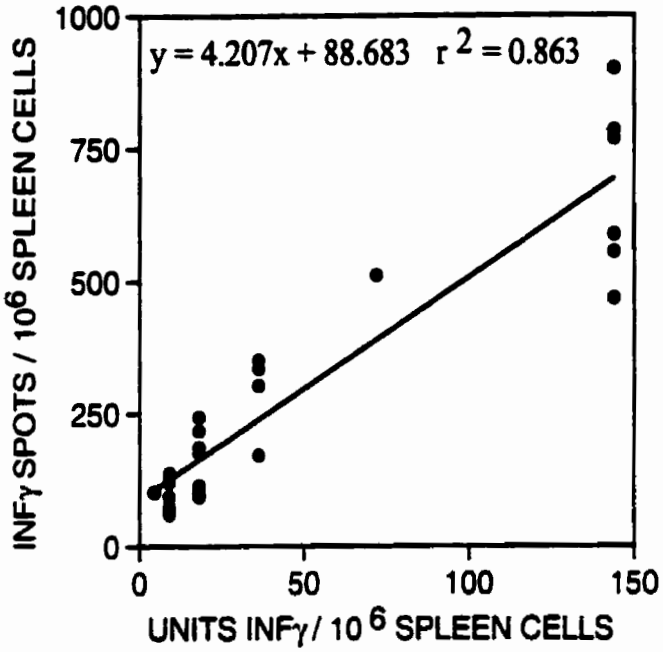



**Figure 3-5** Correlation of the number of IFNγ-producing cells detected by the ELISPOT assay vs. the units of IFNγ detected by the viral cytopathic reduction assay. Mice were immunized with BCG and the assays were performed as described in Methods and Materials. The ELISPOT assay was performed with a total of $10^6$ cells per well. Each point represents results from assays run on an individual BCG-immunized mouse.

## 3.3 Discussion

Enumeration of cytokine-producing cells by ELISPOT provides an efficient method for measuring the effector response of a cell population to a specific antigen. I wished to use this technique in the laboratory to determine the actual number of antigen-specific cytokine-producing cells in a given immune compartment such as the spleen. This requires that the assay be performed upon cells immediately after their removal from the animal, without long in vitro restimulation that can lead to antigen-dependent and antigen-independent changes in the T cell population, including alterations in their cytokine-producing profiles. Accordingly, I undertook to define the parameters that are required for optimal sensitivity of the ELISPOT assay when ex-vivo lymphocytes are used.

When spleen cells from immunized mice were stimulated with antigen only during the ELISPOT assay, the sensitivity of the assay varied depending on the number of cells in the well. The highest sensitivity was observed at high cell densities, and the sensitivity decreased progressively as the number of cells/well was reduced. I found that the sensitivity could be maintained when the immune cells were used at low density by supplementing with spleen cells from normal mice. These supplemental cells did not contribute significantly to the number of spots observed because $10^6$ feeder cells alone produced a negligible number of spots when stimulated with antigen.

The results show that the correlation between the assay sensitivity and cell density is due to the requirement for adequate antigen presentation. The close cell to cell contact that occurs at high cell density allows for intimate contact between the T cells and antigen-presenting cells providing efficient antigen presentation and stimulation. Previous assays have used in vitro antigen-restimulation for varying intervals before the cells were used in the ELISPOT to increase the sensitivity of the assay ( Hutchings et al., 1989; Xu et al., 1991; Muller et al., 1994; Sarawar and Doherty, 1994; De Franco et al., 1995; Milligan and Bernstein, 1995). This method eliminates the need for this additional step by

incubating the cells in the ELISPOT assay at high density, and I believe that the results obtained by this method represent the *in vivo* situation more accurately. Furthermore, by eliminating the necessity for the additional step, this direct assay reduces time and cost.

The assay described is also advantageous when the number of immune cells that can be used must be restricted. If the immune response in the animal is very high, the assay must be performed using fewer immune cells/well so that the spots can be counted reliably. Alternatively, when the assay is being performed on peripheral blood lymphocytes or on cells from murine lymph nodes, then the number of immune cells available may be limited. In each of these situations, spleen cells from syngeneic mice can be added to increase the density of cells/well, thereby maintaining the sensitivity of the assay even when fewer than an optimal number of immune cells is used.

These findings indicate that the current ELISPOT protocol offers an efficient, convenient and very sensitive alternative to other ELISPOT methods, and I have identified efficient antigen-presentation as crucial requirement in establishing maximal sensitivity in any ELISPOT technique. Furthermore, the parameters needed to achieve optimal sensitivity seem to be virtually identical in the different antigen systems employed, namely BCG, leishmania parasites and xenogenic red blood cells (XRBC) (Power et al., 1999).

# 4 Immunization of Mice with a Low Dose of BCG Induces A Th1 Response that is Stable upon Challenge with a High Dose of BCG

## 4.1 Introduction

The influence of immunizing dose has been documented in other antigen systems, both with non-replicating (Salvin, 1958; Parish, 1972; Hernandez-Pando et al., 1997; Ismail and Bretscher, 1999) and replicating antigens (Bretscher et al., 1992; Menon and Bretscher, 1998), demonstrating that low doses in these systems initiated Th1 responses while high doses initiated mixed Th1/Th2 or predominantly Th2 responses. Never the less, it is not generally accepted that antigen dose can be a major factor in determining the type of immunity that is generated in response to an antigen. The experiments detailed below were performed to determine how the dose of BCG administered to mice via the i.d. and i.v. routes of administration influences the Th1/Th2 nature of the immune response. Two types of experiments were performed. In initial experiments, mice were immunized with BCG and the primary immune response to BCG antigen was determined ten wk pi, by ELISPOT assay and by EIA for antibody production. In the second group of experiments, the ability of low dose BCG immunization to establish immune deviation towards the Th1 pole was examined in mice subsequently challenged with a high dose of BCG.

## 4.2 Results

Figures 4-1 and 4-2 and Table 4-1 show the response to primary infection with various doses of BCG administered by the i.d. (Fig. 4-1 and 4-2) and the i.v. route (Table 4-1).

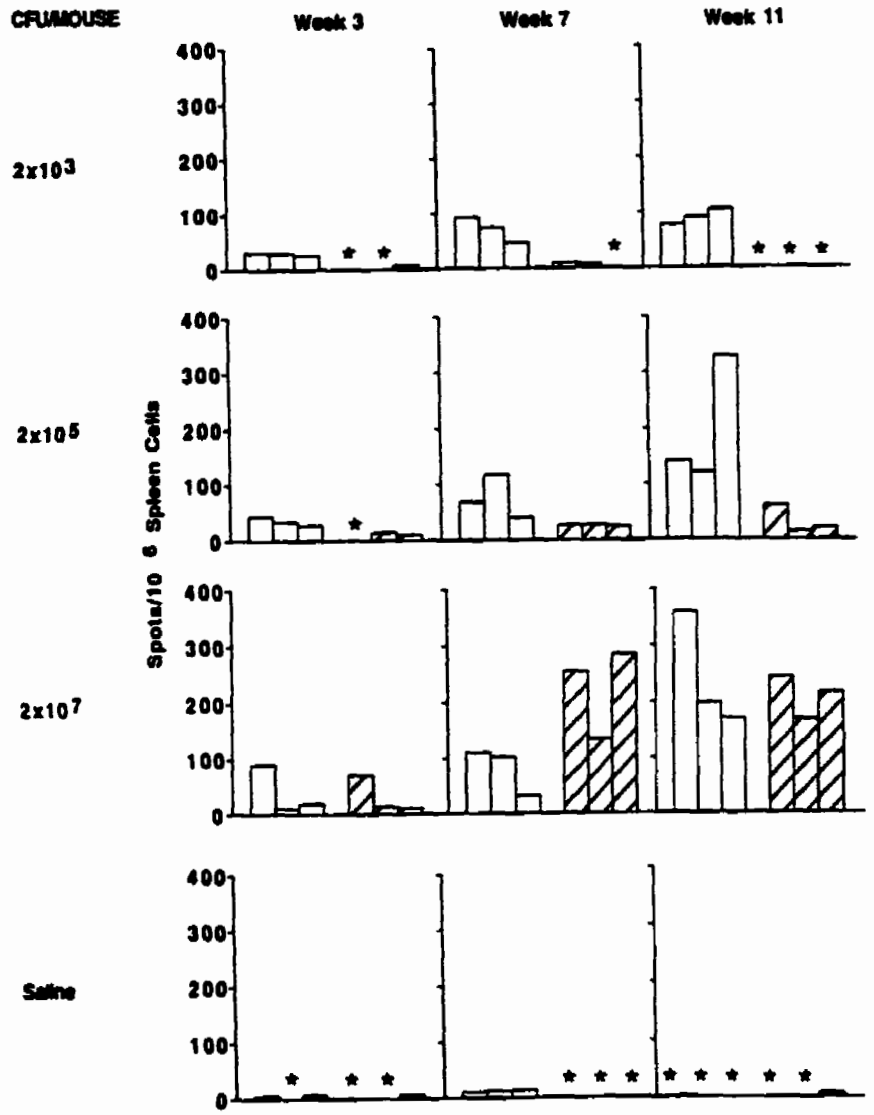



**Figure 4-1** Numbers of BCG-specific cytokine-producing cells/$10^6$ spleen cells as measured by ELISPOT in mice immunized intradermally with different numbers of BCG cfu. Spleens of individual mice were assayed at the indicated times post-infection. Open bars represent the number of IFNγ spots and hatched bars of IL-4 spots. Control mice received saline only. * Indicates number of spots < 5 per $10^6$ spleen cells.

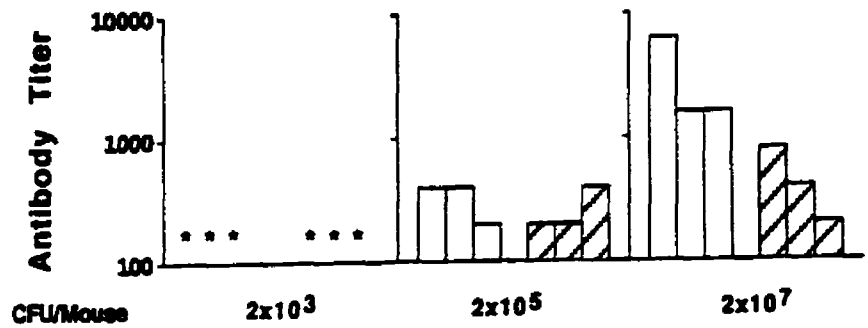



**Figure 4-2** Serum antibody titers (IgG1 and IgG2a) of mice 11 weeks after i.d. immunization with varying doses of BCG. Open bars represent IgG1 and hatched bars IgG2a titers. * Indicates an antibody titer of 100 or less. Negligible titers were observed at 3 weeks post-inoculation. Only immunization with the highest dose induced significant antibody at 7 weeks post-infection. Sera of mice were tested up to 11 months post-infection and no BCG-specific antibody was detected in the sera of mice which received $2x10^3$ BCG during this time.

**Table 4-1** Immune response for individual mice 11 wk after immunization by the i.v. route with different numbers of viable BCG mycobacteria.

| | Cytokine-producing Cells[b] Per $10^6$ Spleen Cells | | Serum Antibodies[c] | |
|---|---|---|---|---|
| Mouse | IFNγ | IL-4 | IgG1 | IgG2a |
| C1[a] | 4 | 2 | 200 | * |
| C2 | 2 | 6 | * | 400 |
| C3 | 2 | 0 | * | 200 |
| C4 | 4 | 8 | * | 200 |
| L1 | 292 | 4 | * | * |
| L2 | 98 | 0 | * | * |
| L3 | 101 | 6 | 100 | 100 |
| L4 | 188 | 0 | 200 | * |
| L5 | 98 | 14 | 200 | 100 |
| H1 | 246 | 222 | $8x10^4$ | $3.2x10^5$ |
| H2 | 212 | 278 | $4x10^4$ | $1.6x10^5$ |
| H3 | 332 | 302 | $8x10^4$ | $3.2x10^5$ |
| H4 | 326 | 300 | $4x10^4$ | $3.2x10^5$ |
| H5 | 398 | 370 | $8x10^4$ | $3.2x10^5$ |

[a] Mice were given saline (C), approx. $2x10^2$ BCG cfu (L), or approx. $2x10^6$ BCG cfu (H).

[b] The ELISPOT assay was used to quantitate cytokine-producing cells.

[c] Antibody titers were determined by enzyme immunoassay and the titer is defined as the last dilution to give a positive result. Prebleed sera had titers of < 100.

* Antibody titer <100.

In the experiment shown in Figure 4-1, groups of mice were immunized with three different doses of BCG; $2x10^3$, $2x10^5$, and $2x10^7$ administered intradermally. A control group was given only saline. The immune response in the spleens of three mice from each group was determined by the ELISPOT assay at 3, 7, and 11 wk post-infection. Fig 4-1 demonstrates that the low dose of $3x10^3$ BCG administered i.d. induced T cells producing IFNγ, but few IL-4-producing cells. The intermediate dose resulted in some IL-4-producing cells, but the IFNγ-producing cells still predominated. In mice that received the highest dose, strong and equally potent IL-4 and IFNγ responses were observed in all three mice. These results demonstrate that while high doses of BCG induce both IFNγ- and IL-4-production by T cells, low doses induce only IFNγ-producing cells.

The IgG1 and IgG2a subclasses of antibody were also examined in the same mice. These two subclasses of antibody were chosen for examination because they are indicative of different types of immune response. IL-4 and IFNγ have differential effects on antibody heavy chain class switching with IL-4 promoting switching to IgG1 and IFNγ favouring IgG2a production (Coffman et al., 1993). As seen in Figure 4-2, the lower dose of BCG did not induce antibody production in any of the three mice at 11 wk pi while the higher dose induced a strong antibody response at 11 wk pi.

Table 4-1 shows similar results upon primary i.v. infection with BCG. In this experiment, mice were given saline or either a low dose or a high dose of BCG, by i.v. injection in the tail vein. Although, on average, the IFNγ response was strongest in the high dose immunized mice, IL-4 was equally high in this group. However, the low dose immunized mice had a significant IFNγ response without the high IL-4 response seen in the high dose immunized mice. Once again, in the mice that received the higher dose of

mycobacteria, there was a very strong antibody response while low dose immunized mice and the controls had very little anti-BCG antibody. G. Wei, using low dose vaccination by the s.c. route, has achieved results similar to those shown here (Power et al., 1998).

These results show that the immunizing dose has a significant impact on the immune response generated to BCG. Further experiments were designed to determine the stability of the immune response generated upon primary exposure to a low dose of mycobacteria, when the same mice were challenged with a significantly higher dose which induces a Th2 response in previously unexposed mice. Groups of mice were injected with different doses of BCG intradermally on the abdomen. A control group was injected similarly with saline. After eight months, the mice were challenged with a higher dose of BCG, one which normally induces a mixed Th1/Th2 response in normal mice. Ten weeks later, mice were killed and the BCG-specific cytokine response in the spleens of mice was determined by ELISPOT assay. Figure 4-3 shows the results of this experiment. Mice that initially had been given lower doses of BCG showed a predominant IFNγ response even upon challenge with the high dose. However, those mice that had been given saline or the higher doses of BCG originally, had a mixed response upon challenge with the higher number of BCG. Similar results have been achieved by G. Wei after s.c. vaccination with BCG (Bretscher et al., unpublished observations). The BCG-specific antibody titers as determined by EIA are presented in Figure 4-4. In this case, most mice had an antibody response after the challenge. However, the IFNγ-dependent IgG2a antibodies predominated in the mice that received lower vaccine doses, while IgG1 predominated in those mice that were given higher doses initially. The results presented in Figures 4-3 and 4-4 are important in that they demonstrate that low dose vaccination is not only capable of inducing a Th1 response, but that under appropriate conditions, this Th1 response is maintained even under conditions that would normally favour a mixed Th1/Th2 response.

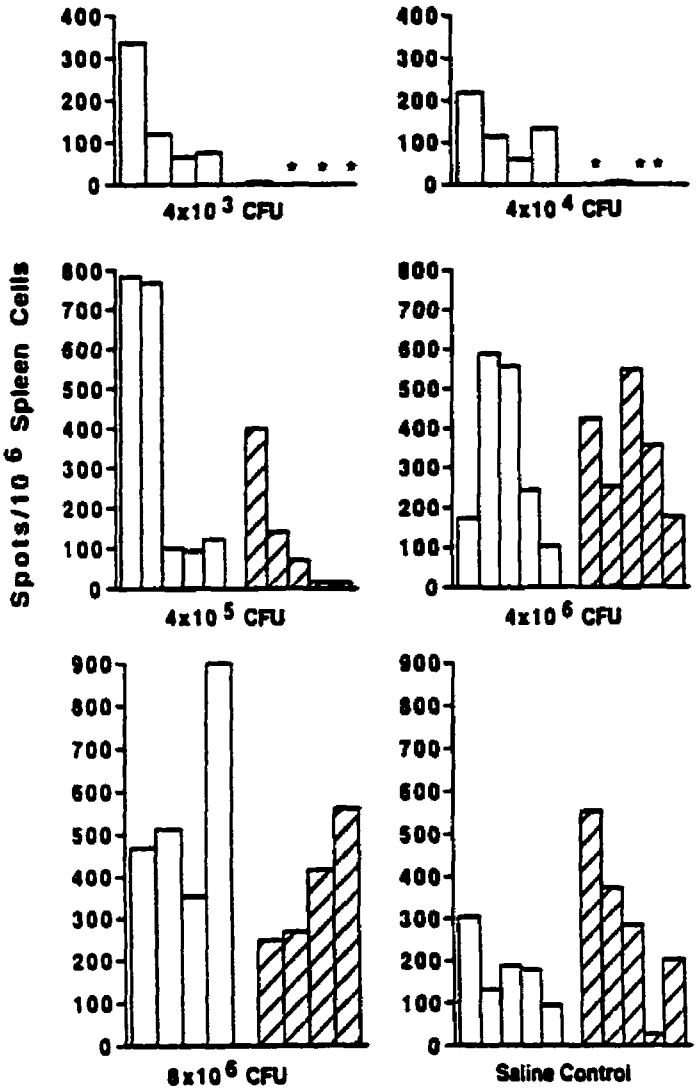



**Figure 4-3** Number of IFNγ- and IL-4-producing BCG-specific cells present in the spleen of mice 10 weeks post-challenge with a high dose of BCG (2 x$10^6$) given intradermally. Each group of mice had been pre-exposed 8 months earlier to a different dose of BCG administered intradermally as indicated in the abscissas. Open bars represent IFNγ spots and hatched bars IL-4 spots of individual mice. * Indicates number of spots < 5 per $10^6$ spleen cells.

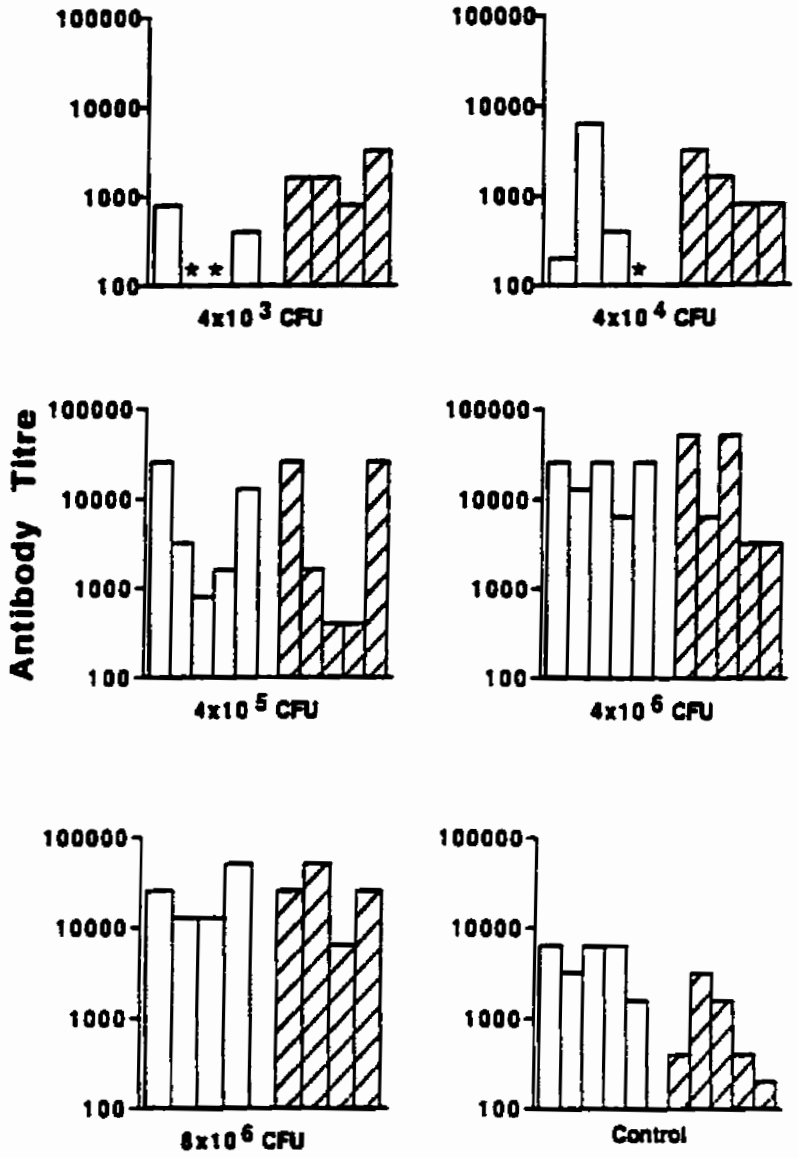



**Figure 4-4** Antibody titers in normal mice and mice pre-exposed to BCG intradermally, 10 wk following an i.d. challenge with a high dose (2 x$10^6$) of BCG. Each group of mice had been pre-exposed 8 months earlier to a different dose of BCG administered intradermally as indicated in the abscissas. Open bars represent IgG1 and hatched bars IgG2a titers. * Indicates titer of 100 or less.

It should be noted that unimmunized mice sometimes exhibit a weak antibody response to BCG as they age. I believe that these mice have been exposed to mycobacterial species in their environment and that antibodies produced in response to these environmental mycobacteria cross-react with the BCG antigen used in the EIA. Indeed, the TB lab of the Department of Microbiology and Immunology has isolated a saprophytic mycobacterial species, *M. gordonii* from samples of the drinking water of mice in the animal colony. I compared immunoglobulin titers in mice immunized with a low dose of BCG with those of unimmunized control mice. The results are shown in Table 4-2. Although the distinction is not absolute, the data suggest that the low dose BCG vaccination has had an effect in reducing the both the number of animals seroconverting, as well as the amount of antibody produced by these animals.

**Table 4-2** Seroconversion rates and antibody titers to mycobacterial antigens in low dose BCG-immunized and control mice.

| Experiment | Treatment | No. Seropositive[b] | Mean Titer of Seropositives |
|---|---|---|---|
| 1 | Control | 11/16 | 400 |
| | Low dose[a] | 5/16 | 250 |
| 2 | Control | 4/10 | 1600 |
| | Low dose | 1/10 | 400 |

[a] Mice received approx. $4x10^3$ BCG by i.d. injection. All mice were 6-8 weeks of age at the time of immunization and serum antibody was measured by enzyme immunoassay at 10-16 weeks post-immunization. [b] The probability of seroconversion was significantly greater among the control mice than among the low dose vaccinated mice (Chi-square value = 6.47, DF = 1, $p < 0.02$)

## 4.3 Discussion

These results show the significant impact which vaccine dose has on the development of the immune response to BCG. Although there are many variables that can contribute to the determination of Th cell fate and thus the nature of the immune response to mycobacteria, I believe that controlling a single variable, namely dose, will reduce or eliminate the influence of many other variables. Consider the factor of genetic variability in a population. Different strains of mice mount very different types of immune responses to the same dose of BCG antigen. BALB/c mice generate a Th2 response at a much lower dose than C57BL (Power and Bretscher, unpublished observations). Individuals among the human population, like inbred mouse strains, are likely to show similar differences due to genetic diversity. This seems to be an insurmountable problem. How can we ensure an effective immune response in all individuals regardless of genetic diversity? A solution to this problem has been proposed by Bretscher (1992b). This hypothesis is based on the ability of live BCG organisms to replicate once injected as a vaccine. A suitably low dose of BCG will induce a Th1 response in those individuals that are prone to respond to such a low dose. In other individuals, a low dose will not induce any immune response initially, but because the organism is alive and capable of replicating in the host, it will eventually reach a dose which will be detectable by the immune system, at which point it will induce a Th1 response. Thus, by immunizing with a suitably low dose of BCG, it may be possible to induce an appropriate Th1 response in the majority of individuals vaccinated, regardless of their genetic predisposition.

Another factor that has the potential to interfere with the efficacy of BCG vaccination is previous exposure to environmental mycobacteria. Immune priming to mycobacterial antigens that cross-react with BCG has the potential to affect the response to subsequent BCG vaccination or exposure to *M. tuberculosis*. In the mouse colony, I have noticed that it is more difficult to establish immune deviation in older mice.

Furthermore, dose dependence to primary BCG vaccination is not as clear in older mice. I hypothesize that this is due to exposure of these mice to mycobacteria in their environment that establishes a non-exclusive Th1 response (i.e., having a significant Th2 component) and subsequently prevents the low dose vaccine from establishing a Th1 response. Based on the findings presented, I hypothesized that a low dose vaccine given at an early age may establish a Th1 response that subsequently prevents the development of a Th2 response upon natural exposure to environmental mycobacteria. If this were the case, I expect to see fewer of the mice given the low dose BCG vaccination developing antibody titers to BCG antigen than in the control group. Indeed, this is what we observed. The fact that low dose immunization at an early age seems to prevent seroconversion of mice naturally exposed to mycobacteria is a significant finding, in that it suggests an approach by which the negative influence of such exposure could be overcome. Because early exposure to BCG administered parenterally to mice reduces the chance of them developing a possibly harmful Th2 response, vaccination of infants with low doses of BCG may be a mechanism by which the negative influence of environmental mycobacteria could be eliminated or reduced. Establishing a beneficial immune imprint before the negative effect of exposure to environmental mycobacteria can occur may offer a solution to the problem. These results support neonatal BCG vaccination as an approach to circumvent any negative influences of exposure to environmental mycobacterial.

It remains to be determined whether low dose vaccination with BCG will provide greater protection than the standard dose, or whether i.d. immunization can provide protection by natural challenge via the respiratory route. Many lines of evidence suggest that it will. In animal studies, BCG administered by parenteral routes provides some protection to respiratory challenge with virulent *M. tuberculosis*, indicating that this method of immunization provides a degree of protection at mucosal sites. Secondly, some studies in humans have shown that BCG offers a high rate of protection from

development of TB after natural exposure to *M. tuberculosis* (Aronson et al., 1958; Ferguson, 1955; Hart and Sutherland, 1977). Thus, the standard means of vaccination is capable of inducing effective immune response at the site of infection at least in some populations. Finally, in studies in cattle, low dose immunization given subcutaneously was able to provide significant protection from challenge that mimicked natural infection (Buddle et al., 1995a; 1995b). The results of these studies, together with the immunological evidence presented here, indicates that effective low dose vaccination of humans is a possibility. The Saskatchewan Research Centre for Elimination of Tuberculosis is initiating a human trial of the efficacy of neonatal low dose BCG vaccination here in Saskatchewan.

I have previously suggested that individuals may develop TB as a result of two distinct types of failure by the immune system. Although this may be the case, low dose vaccination would be appropriate for individuals prone to develop either type of disease. For those who would develop TB as a result of insufficient Th1 immunity, a low dose vaccine would induce an appropriate response so that upon exposure to *M. tuberculosis*, a stronger Th1 and more rapid response would be induced before the infection could spread. For those prone to develop a Th2 response upon infection with *M. tuberculosis*, Th1 imprinting with low dose BCG vaccination would prevent the development of the damaging Th2 response. A single vaccination protocol would be appropriate for both groups, eliminating the necessity to distinguish between them.

Antigen load or dose is not a static parameter, and is dependent on several variables. For non-replicating organisms such as killed organisms, xenogenic red blood cells, or protein antigens administered by an immunologist, the administered dose alone determines the immune response. For live vaccines, another factor comes into play: the replication rate of the organism once it is introduced into the vaccinated host. For relatively slow growing organisms such as leishmania parasites and BCG, the replication rate is not a problem, and small differences in replication rate between strains can be

overcome by varying the immunizing dose. However, in the case of more rapidly dividing organisms such as *Plasmodium* sp., the replication rate is a concern. In this case, regardless of the number of organisms administered, the rapid growth of the parasite results in a large number of organisms within a very short time (Taylor-Robinson and Phillips, 1998). This *effective* dose results in a Th2 response regardless of the immunizing dose. Thus, the low-dose vaccination strategy will be an effective method for achieving a Th1 response only when it is used with non-replicating or slowly replicating antigens.

In most natural infections, the replication rate of the organism is likely to be the most prominent determinant of antigen load and hence of the resulting immune response, as initial infections are likely to result from limited numbers of organisms. In an interesting set of experiments, North and Izzo demonstrated that one indicator of mycobacterial virulence is the initial in vivo replication rate. All virulent strains of mycobacteria tested had an initial rapid rate of replication in vivo while avirulent BCG did not (North and Izzo, 1993). Therefore, it is possible that a rapid in vivo replication rate is important for mycobacterial pathogenicity by quickly establishing a Th2 response so that the mycobacterial species can escape effective immune control.

The results of experiments presented here have also shown that low doses of BCG can generate an "immune imprint" such that, upon challenge with a dose that normally induces Th2 cells, a predominant Th1 response is maintained. This immune deviation is a requirement if such a vaccination strategy is to have any utility for vaccinating against organisms that are best contained by cell-mediated immunity. If the protective Th1 response is not maintained upon natural challenge with the pathogen, then the vaccine will not be effective. Therefore, it is an important observation that a low dose of BCG not only induces a Th1 response, but given sufficient time between vaccination and challenge, such an immune response becomes stable so that it can resist the influence

of subsequent conditions which would normally lead to the development of a humoral response.

The development of a stable immune response requires a considerable amount of time between vaccination with BCG and subsequent high dose challenge (3-4 months for BCG Montreal strain in BALB/c mice), before the immune response upon challenge is locked into a Th1 mode. Challenging mice with a high dose before this time does not result in immune deviation, and the immune response is of a mixedTh1/Th2 or predominant Th2 type (unpublished observation). I believe that the time required for establishment of stability in the immune response is necessary for the development of antigen-specific cells that can actually inhibit the antibody response. Current theories suggest that a simple Th1/Th2 dichotomy is responsible for establishing the exclusivity of the response. Once a strong Th1 response is established, Th1 cytokines such as INFγ inhibit the generation of cells of the Th2 type, and vice versa. However, a study by Ramshaw et al. (1977) found that under conditions that favour generation of a DTH response, $CD8^+$ cells were generated that had the capacity to suppress antibody responses to the same antigen upon adoptive transfer to syngeneic mice. These results suggested that $CD8^+$ cells may be involved in establishing, and maintaining the stable Th1 response and suppressing the Th2 response at the time of challenge.

# 5 Characterization of the Immune Response to a Foreign Protein Expressed in BCG

## 5.1 Introduction

This chapter examines the immune response in mice immunized with rBCG expressing the β-gal protein. The purpose of this work was to evaluate the potential use of low dose vaccination with rBCG as a method to immunize against diseases for which a Th1 response is protective. β-gal was chosen as a candidate protein because it is known to be expressed in mycobacteia at levels which can induce an immune response. Furthermore, purified β-gal is easily obtained from a commercial source at a reasonable cost and anti-β-gal antibodies could also be easily obtained. Mice were immunized with various doses of rBCG expressing the β-gal protein or with a high dose of BCG transformed with a control vector containing the gene for the HIV gag protein. The immune response to BCG antigen and to the β-gal protein were assessed by ELISPOT and ELISA at 10wk post immunization. To determine the stability of the immune response to the recombinant BCG, some mice were challenged with a high dose of rBCG 16 wk after the initial immunization and their immune responses were assessed after an additional 16 wk as described in the Materials and Methods section.

## 5.2 Results

It was necessary to ensure that β-gal was indeed expressed in the mycobacteria that were transformed with the pMV361::*lacZ* plasmid. The western blot shown in Figure 5-1 demonstrates the expression of *E. coli* β-gal in BCG mycobacteria transfected with the pMV361::*LacZ* plasmid. The protein content of the BCG lysate was estimated at 2000 μg/ml by BCA protein assay, and 10 μl (approx. 20 μg of protein) was loaded. Staining of the β-gal band in lane 1 is estimated to be at least twice the intensity of that in lane 3. Therefore, β-gal expression is expected to be approximately 2-3% of the total protein production in the rBCG.

Figures 5-2 through 5-5 represent a preliminary experiment in which the immune response to rBCG expressing β-gal was assessed. Figures 5-2 and 5-3 show the number of BCG- and β-gal-specific IFNγ-producing cells in the spleens of mice 9 wk after immunization with different doses of rBCG. As predicted, immunization with both low and high doses of rBCG induced not only BCG-specific, but also β-gal-specific IFNγ-producing cells. This is encouraging because it indicates that not only is the β-gal expressed in vivo, but also that expression is at a significant level to induce an immune response. Unfortunately, for this experiment, the results of the IL-4 assay are not available due to a technical problem. However, a surrogate marker for a Th2 response is serum antibody, and as serum was collected, antibody titers were determined for these mice. These results are shown in Figures 5-4. Regardless of the route of immunization, prominent antibody responses were only observed in the high dose immunized mice. I concluded that high doses of BCG induced a mixed Th1/Th2 response to both the BCG

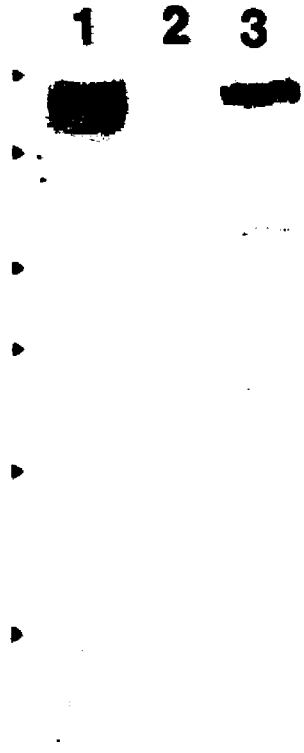



**Figure 5-1.** β-gal is expressed in BCG transfected with pMV361::*LacZ* as detected by immunoblot. Lanes 1 and 2 contain 10 µl of lysate prepared from recombinant BCG expressing β-gal (rBCGβgal) and BCG respectively as described in the Materials and Methods section. The protein content of the sonicate is estimated to be about 2000 µg/ml as determined by BCA protein assay. Lane 3 contains 0.1 µg of a commercial preparation of purified β-gal of approximate molecular weight, 116,000 (Sigma Chemicals). Molecular weight markers are indicated by the arrowheads at the left, from top to bottom: maltose-binding protein-β-gal (175kDa), maltose-binding protein-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triosephosphate isomerase (32.5 kDa), and β-lactoglobulin A (25 kDa).

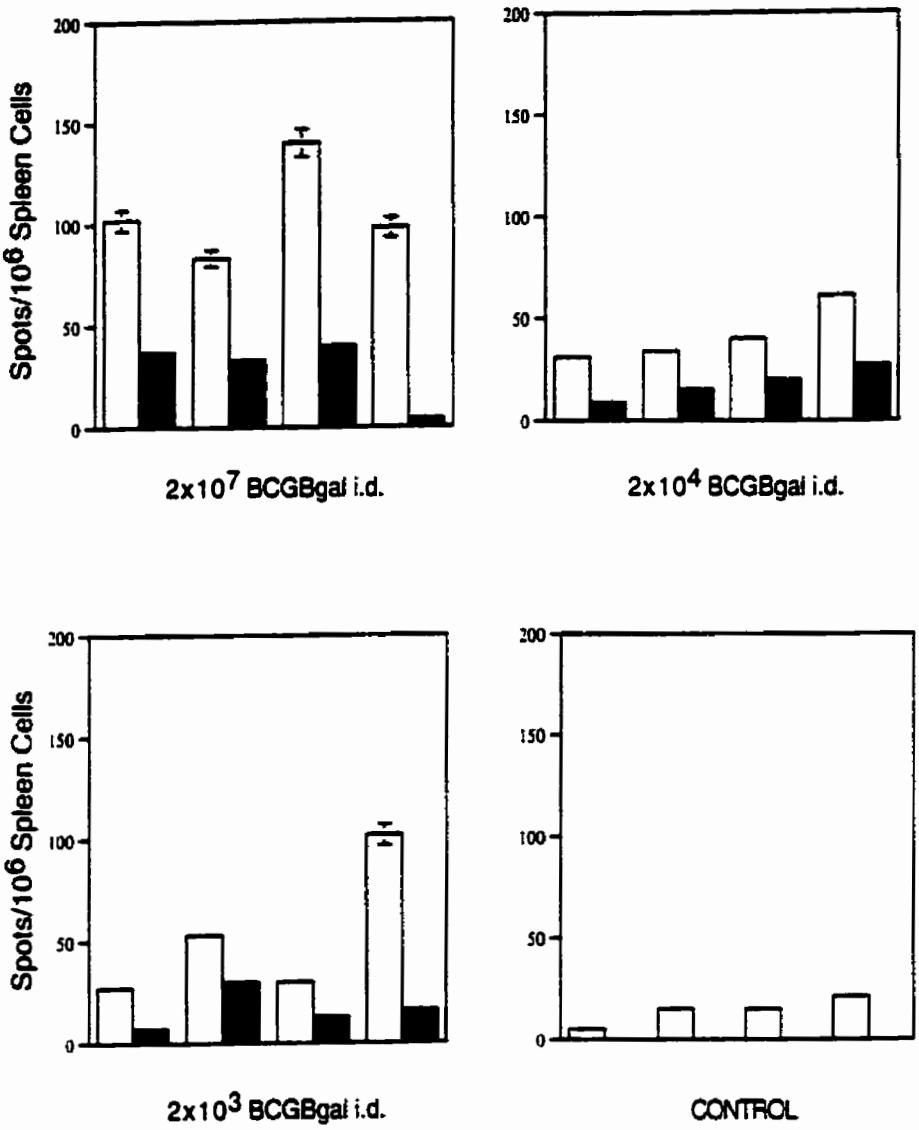



**Figure 5-2.** Number of IFNγ-producing cells in the spleens of individual mice immunized with different numbers of rBCGβgal by the i.d. route at 10 wk post-immunization. The values for saline-injected control mice are also shown. Open bars indicate the number of BCG responsive cells while the black bars represent β-gal responsive cells.

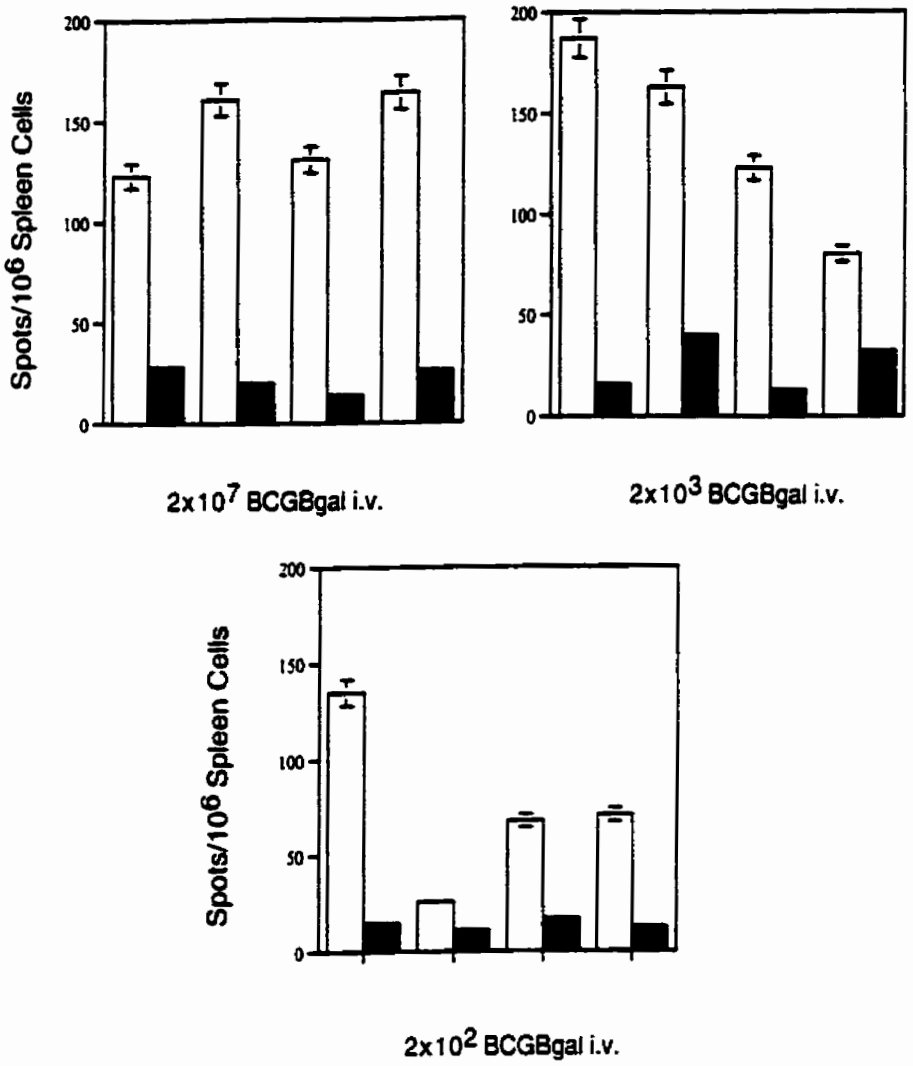



**Figure 5-3.** Number of IFNγ-producing cells in the spleens of individual mice immunized with different numbers of rBCGβgal by the i.v. route at 10 wk post-immunization. Open bars indicate the number of BCG responsive cells while the black bars represent β-gal responsive cells.

itself and to the β-gal, as this dose induced both IFNγ-producing T cells and antibodies to the BCG and the incorporated β-gal. Mice given the lower doses of rBCG did not produce significant amounts of antibodies to either BCG or β-gal, but did have spleen cells that produced IFNγ in response to both of these antigens. These observations indicate that the mice receiving a low dose had only a Th1 response to the BCG as well as to the expressed foreign protein. I proceeded to determine if the Th1 response induced by the low dose of rBCG would be stable upon a subsequent challenge with a high dose of rBCG, which would induce a Th2 response in normal mice. The results of an experiment in which rBCG were given initially by the i.d. route and subsequently challenged by the same route are shown in Figure 5-5. In mice initially immunized with the low dose of rBCG, a predominantly Th1 response is maintained even after the challenge dose is given. In comparison, the control mice that received only the challenge dose have a much more prominent Th2 component to their immune response, as do those mice that were given a high dose of BCG initially. All mice immunized by the i.v. route developed a mixed response in the spleen when challenged with a high dose of rBCG by i.d. injection (data not shown). Thus, it appears that low dose i.d. immunization provides a stable immune response when mice are challenged by this route. Intravenous immunization provides a strong Th1 response when mice are given a low dose vaccination by this route; however, this Th1 response appears to be more easily deviated to Th2 upon challenge by the heterologous route.

To confirm these observations, a second experiment was carried out following a similar protocol. The results of this experiment are given in Figures 5-6 through 5-16. Figures 5-6 and 5-7 show the immune response to different doses of rBCG given by i.v. (Figure 5-6) and i.d. (Figure 5-7) immunization. Once again, regardless of the route, high doses of rBCG induce a mixed Th1/Th2 (both IFNγ and IL-4) response, while

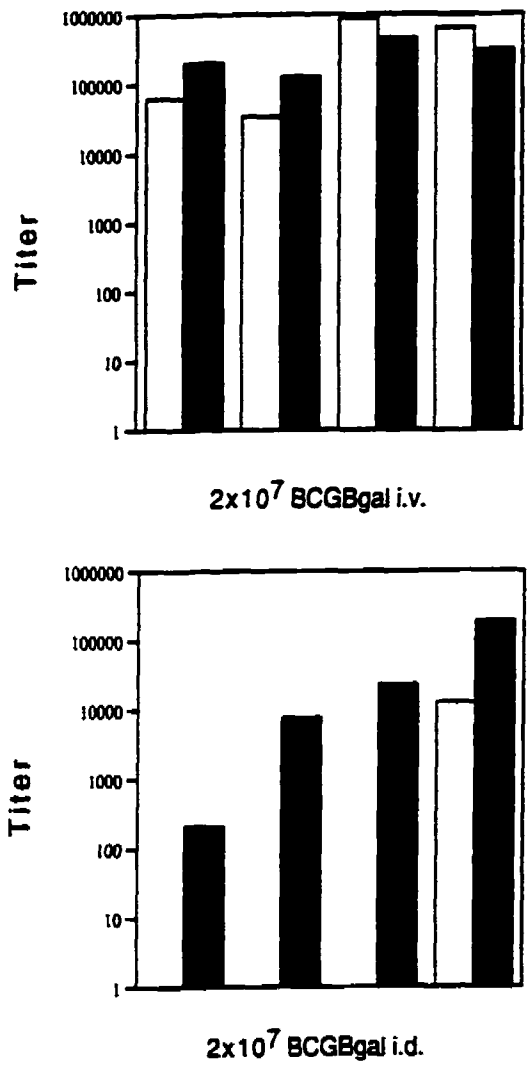



**Figure 5-4.** IgG1 and IgG2a antibody response to β-gal in serum of mice receiving $2x10^7$ rBCG by the i.v. and i.d. routes. These are the same mice depicted in Figures 5-2 and 5-3. Negligible antibody titers were observed in mice receiving lower doses. Antibody titers were determined by enzyme immunoassay. IgG1 (open bars) and IgG2a (black bars)

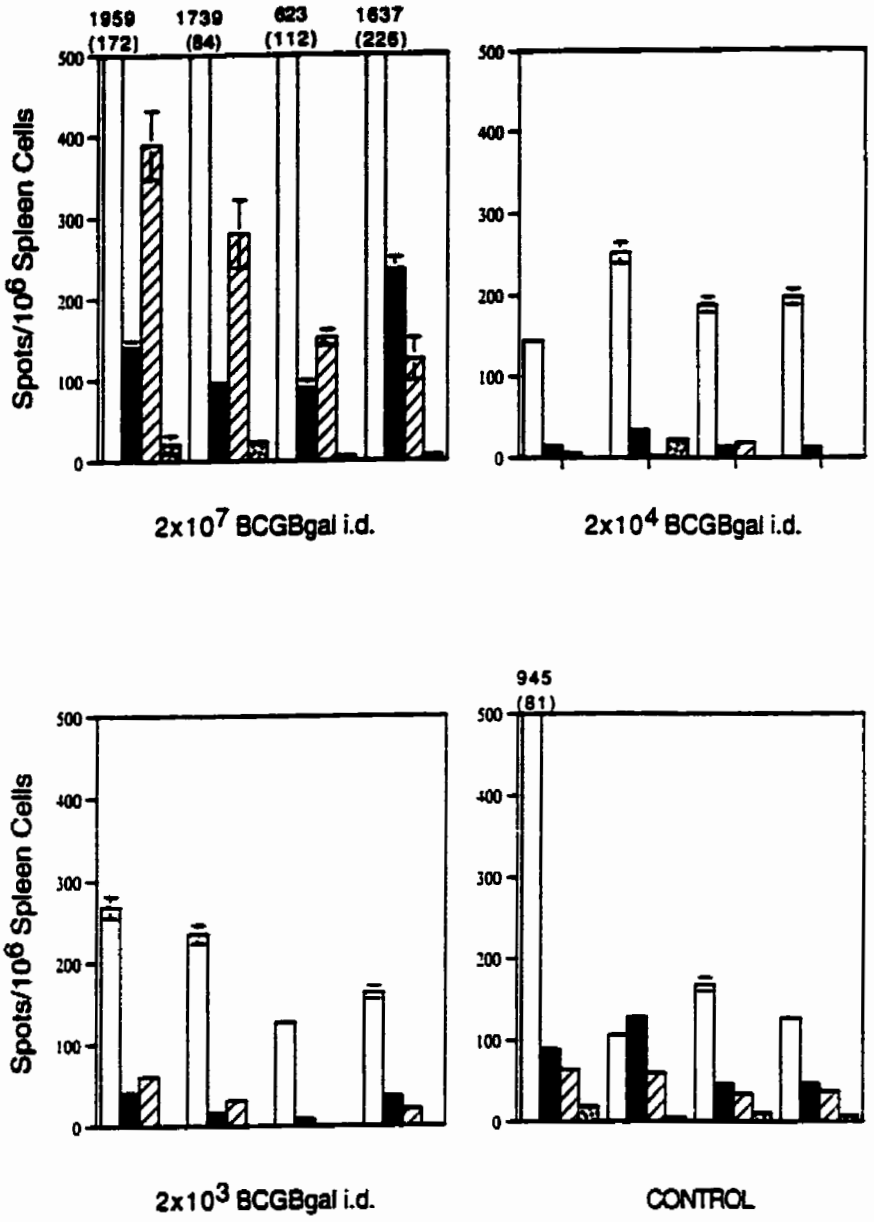



**Figure 5-5.** IFNγ and IL-4 responses to BCG and β-gal of mice immunized with different numbers of rBCGβgal by the i.d. route and then challenged intradermally with a high dose ($2x10^7$) of rBCG. Mice were first immunized with the number of BCG cfu as indicated under each graph. After 4 months, all mice were challenged with the high dose of rBCG. The control group received the challenge dose only. In each case, the number of BCG and β-gal specific IFNγ- and IL-4-producing cells was determined by ELISPOT sixteen wk after administration of the challenge dose. Open bars - IFNγ response to BCG; Black bars - IFNγ response to β-gal; Hatched bars - IL-4 response to BCG; Stippled bars - IL-4 response to β-gal. Where values are off the chart, the value and SD (in brackets) are given above the bar.

lower doses induce a predominantly Th1 (IFNγ) response to both BCG and the expressed β-gal antigen. Mice immunized with BCG transformed with a control vector not expressing β-gal (Figure 5-6) show no response to β-gal while cells from control mice shown in Figure 5-7 do not respond to BCG-lysate antigen or β-gal antigen. Figures 5-8 and 5-9 show the IgG1 and IgG2a titers to β-gal antigen in the same groups of mice. Only mice immunized with the high doses of rBCG have significant titers to β-gal indicating that the antibody response is indeed attributable to immunization with recombinant BCG, and that only higher doses will induce antibody production. For comparison, the antibody responses to BCG antigen in these same mice are included in Figures 5-10 and 5-11. Once again, high doses of BCG are required for antibody production.

Mice from each group were challenged four months after immunization with a high dose of rBCGβgal given i.d. to determine the stability of the Th1 response induced by the low dose vaccination. The immune response was assayed at 16 wk post-challenge, as opposed to 10 wk post-challenge as in previous experiments. Mice were assayed at a later time, not by design, but due to the unavailability of the ELISPOT plates.

Mice that were immunized with low doses by the i.d. route (Figure 5-12) possessed a strong and stable Th1 response to BCG antigen and to β-gal after the challenge with a high dose and the immune profile of these mice was distinct from that of the control mice that received only the challenge dose. However, results from mice that were challenged by the i.d. route after i.v. immunization were perplexing (Figure 5-13). Mice that were given the lowest dose ($2x10^2$) clearly demonstrated no immune deviation as they showed a strong and even dominant IL-4 response, indicating that Th1 immune

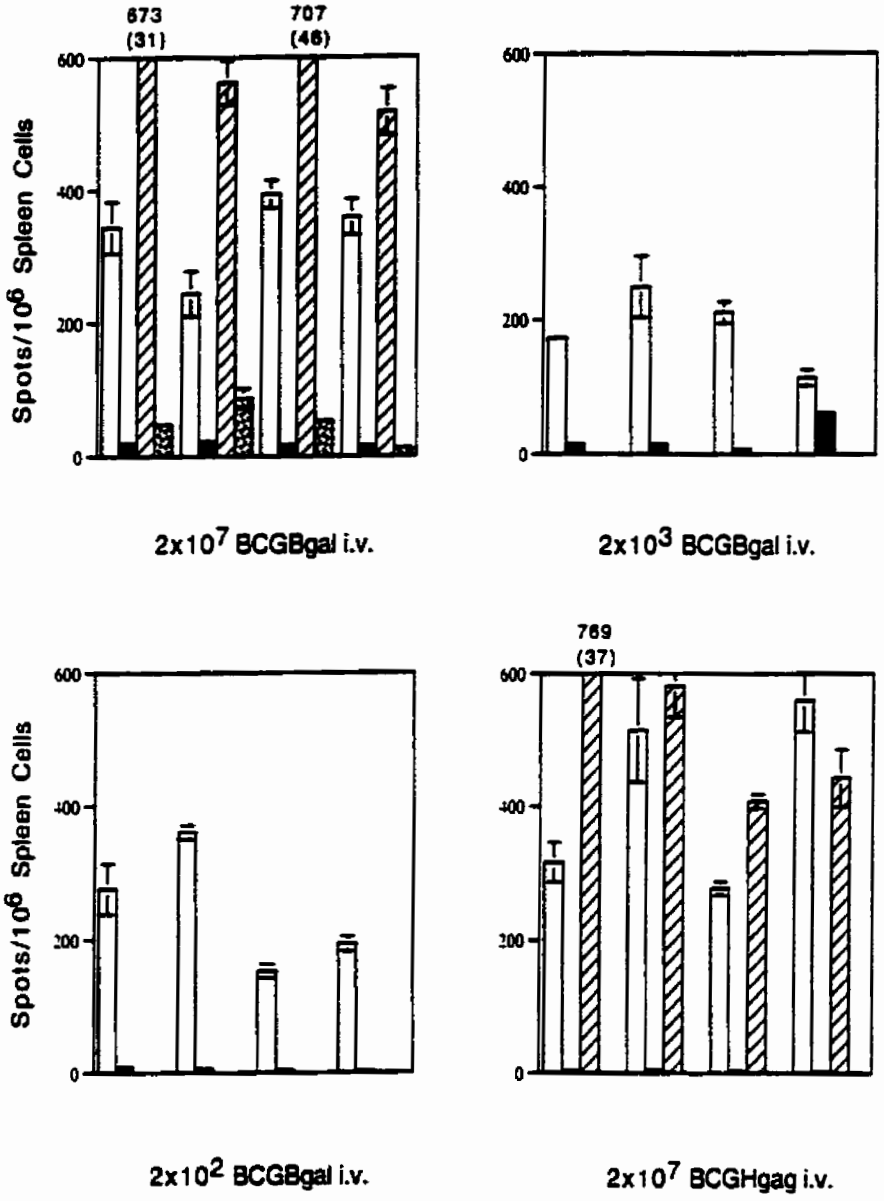



**Figure 5-6.** Immune response of individual mice after i.v. immunization with different doses of recombinant BCG (rBCG). Mice were immunized as indicated under each graph. After 9 wk, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. The response in mice given a high dose of BCG transformed with a control vector containing the HIV gag sequence is also shown. Open bars - IFNγ response to BCG; Black bars - IFNγ response to β-gal; Hatched bars - IL-4 response to BCG; Stippled bars - IL-4 response to β-gal. Where values are off the chart, the value and SD (in brackets) are given above the bar.

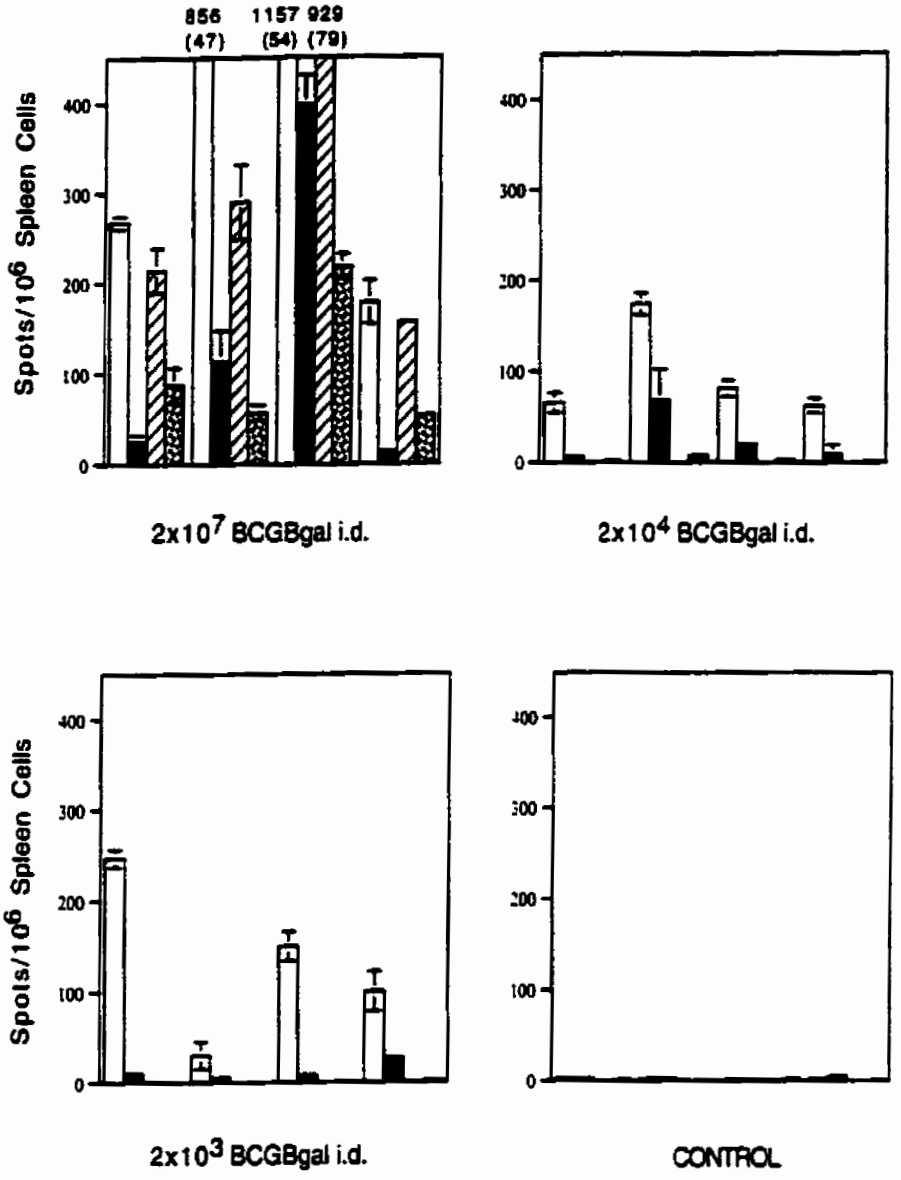



**Figure 5-7.** Immune response of individual mice after i.d. immunization with different doses of recombinant BCG. Mice were immunized as indicated under each graph. After 9 wk, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. The response in unimmunized control mice is also shown. Open bars - IFNγ response to BCG; Black bars - IFNγ response to β-gal; Hatched bars - IL-4 response to BCG; Stippled bars- IL-4 response to β-gal. Where values are off the chart, the value and SD (in brackets) are given above the bar.

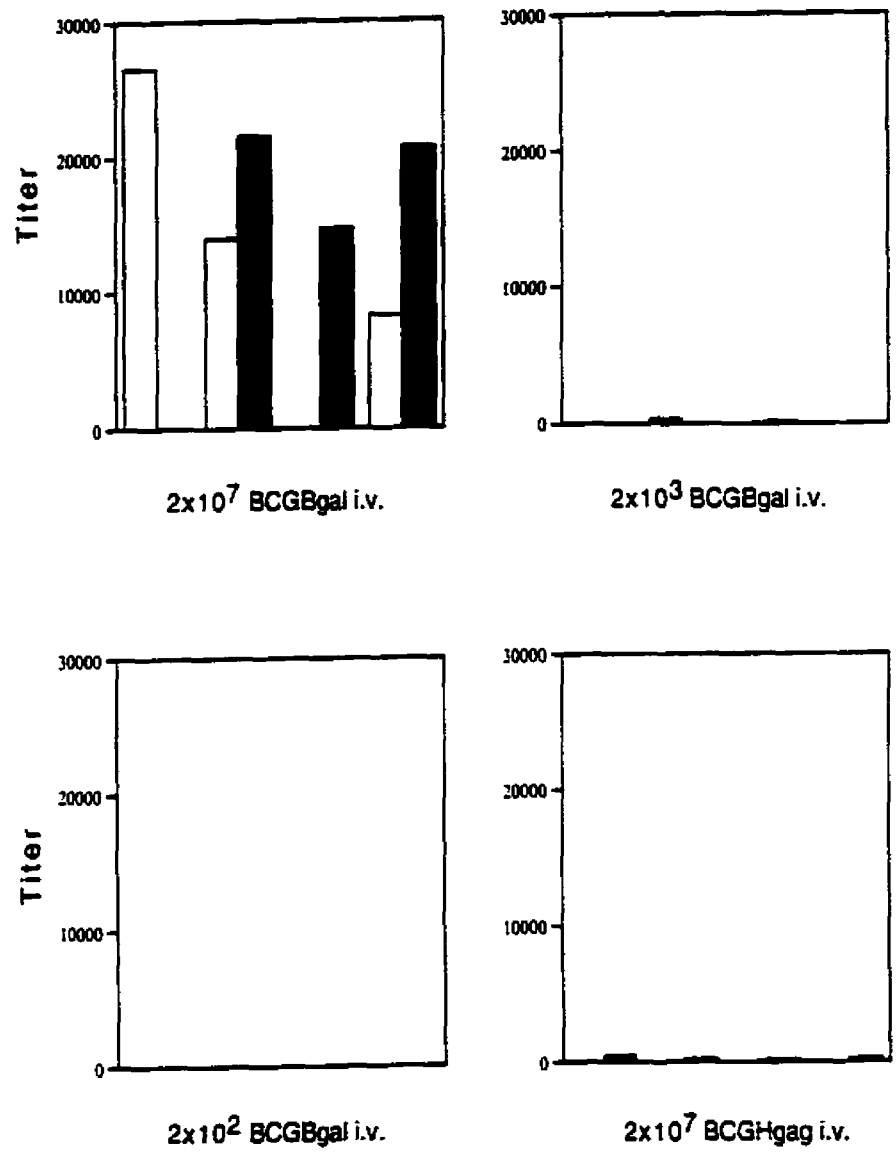



**Figure 5-8.** Serum antibody responses of mice to β-gal 9 wk after immunization with different doses of rBCG by the i.v. route. Results for mice immunized with a high dose of BCG transformed with a control vector are also shown. Open bars show IgG1 titers while black bars depict IgG2a titers.

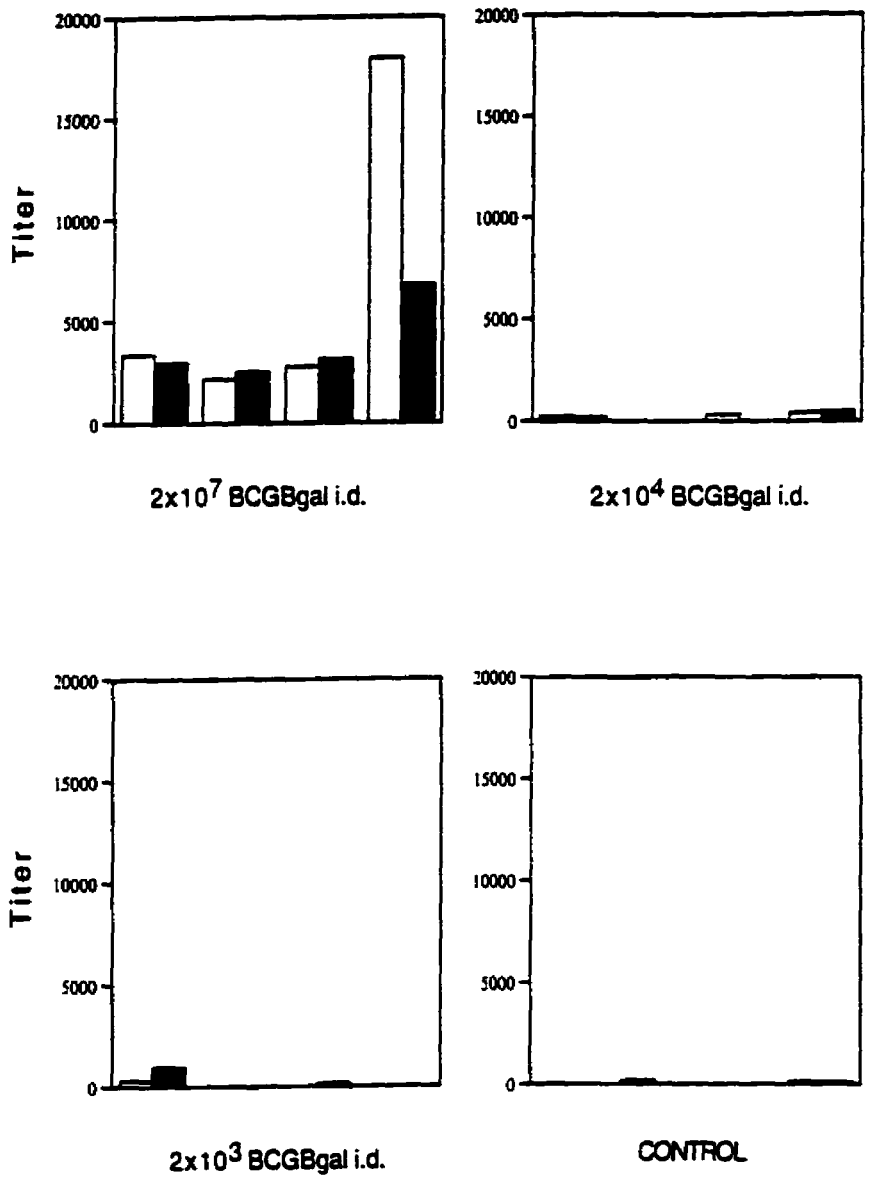



**Figure 5-9.** Serum antibody responses of mice to β-gal 9 wk after immunization with different doses of rBCG by the i.d. route. The results for unimmunized, age-matched control mice are also shown. Open bars show IgG1 titers while black bars depict IgG2a titers.

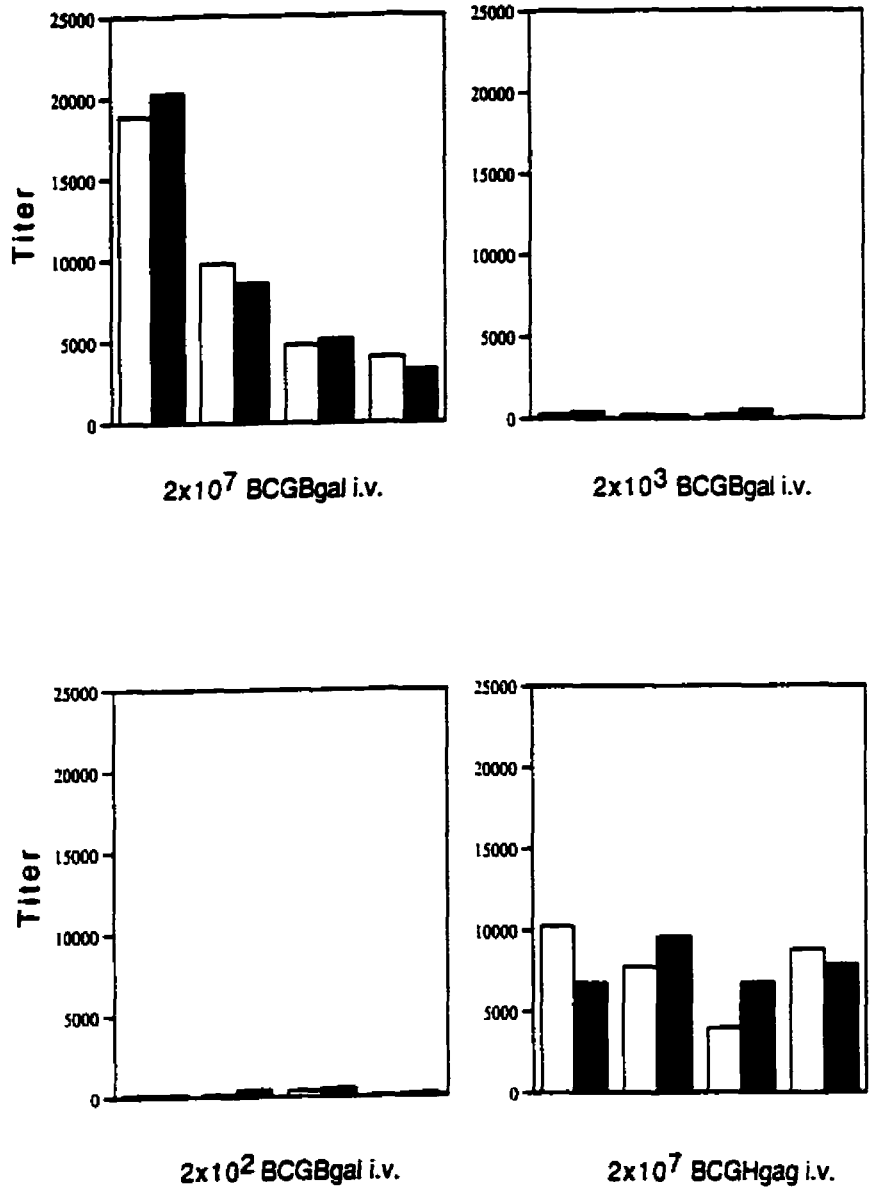



**Figure 5-10.** Serum antibody responses of mice to BCG antigen 9 wk after immunization with different doses of rBCG by the i.v. route. Results for mice immunized with a high dose of BCG transformed with a control vector are also shown. Open bars show IgG1 titers while black bars depict IgG2a titers.

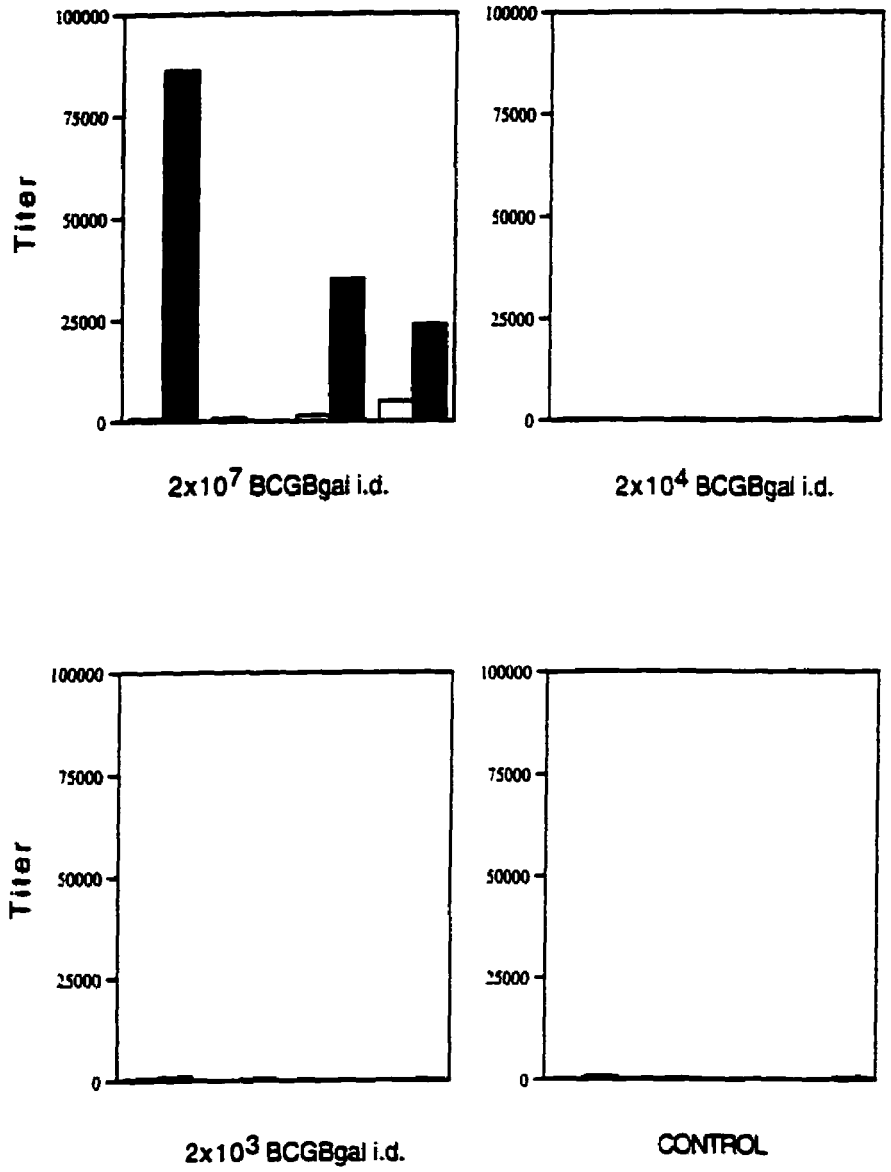



**Figure 5-11.** Serum antibody responses of mice to BCG antigen 9 wk after immunization with different doses of rBCG by the i.d. route. The results for unimmunized, age-matched control mice are also shown. Open bars show IgG1 titers while black bars depict IgG2a titers.

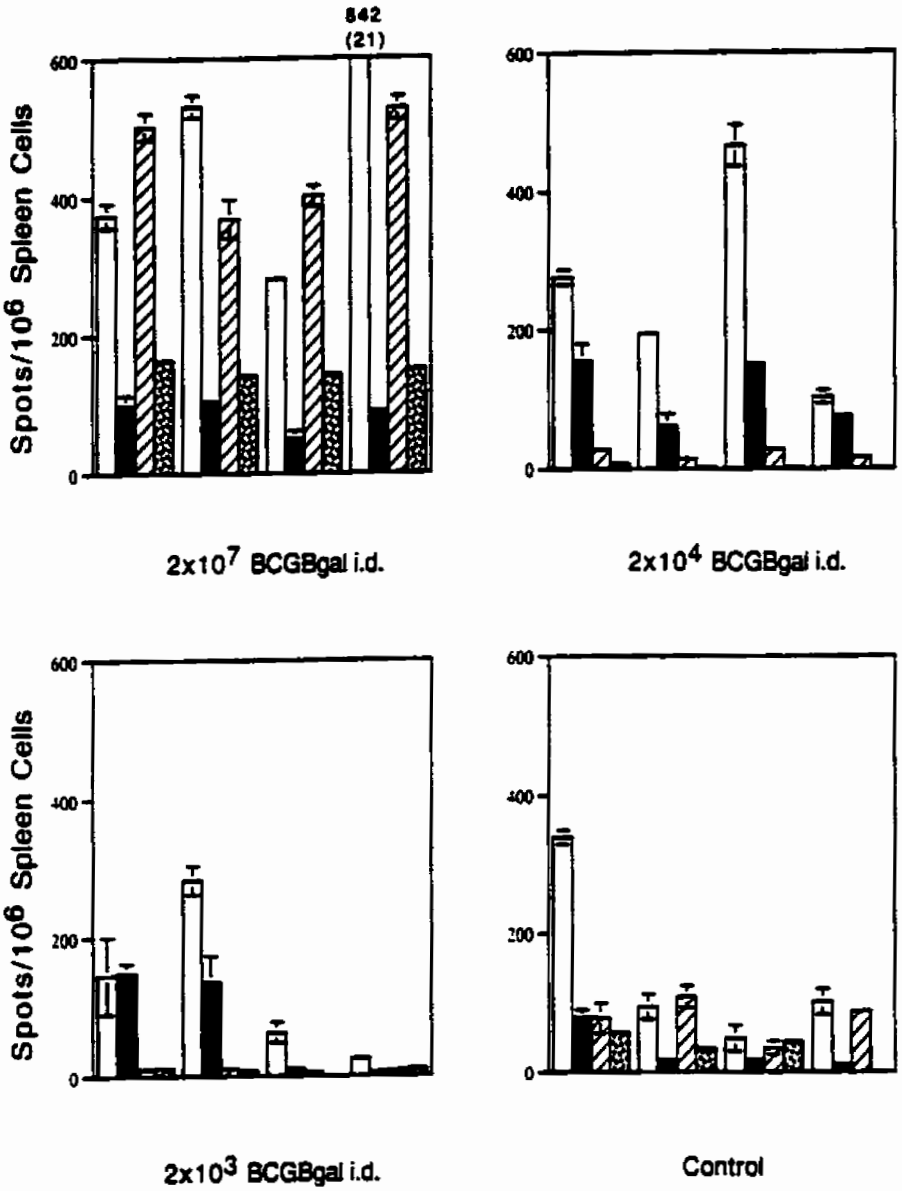



**Figure 5-12.** Immune response of individual mice vaccinated with different doses of rBCGβgal after subsequent i.d. challenge with a high dose of rBCGβgal. Each group of mice was initially given an i.d. vaccination dose of rBCGβgal as indicated under each graph. After 4 months, the mice were challenged with approximately 2 x$10^7$ rBCGβgal cfu given i.d. After an additional 16 wk, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. Control mice received the challenge dose only. Open bars - IFNγ response to BCG; Black bars - IFNγ response to β-gal; Hatched bars - IL-4 response to BCG; Stippled bars - IL-4 response to β-gal. Where values are off the chart, the value and SD (in brackets) are given above the bar.

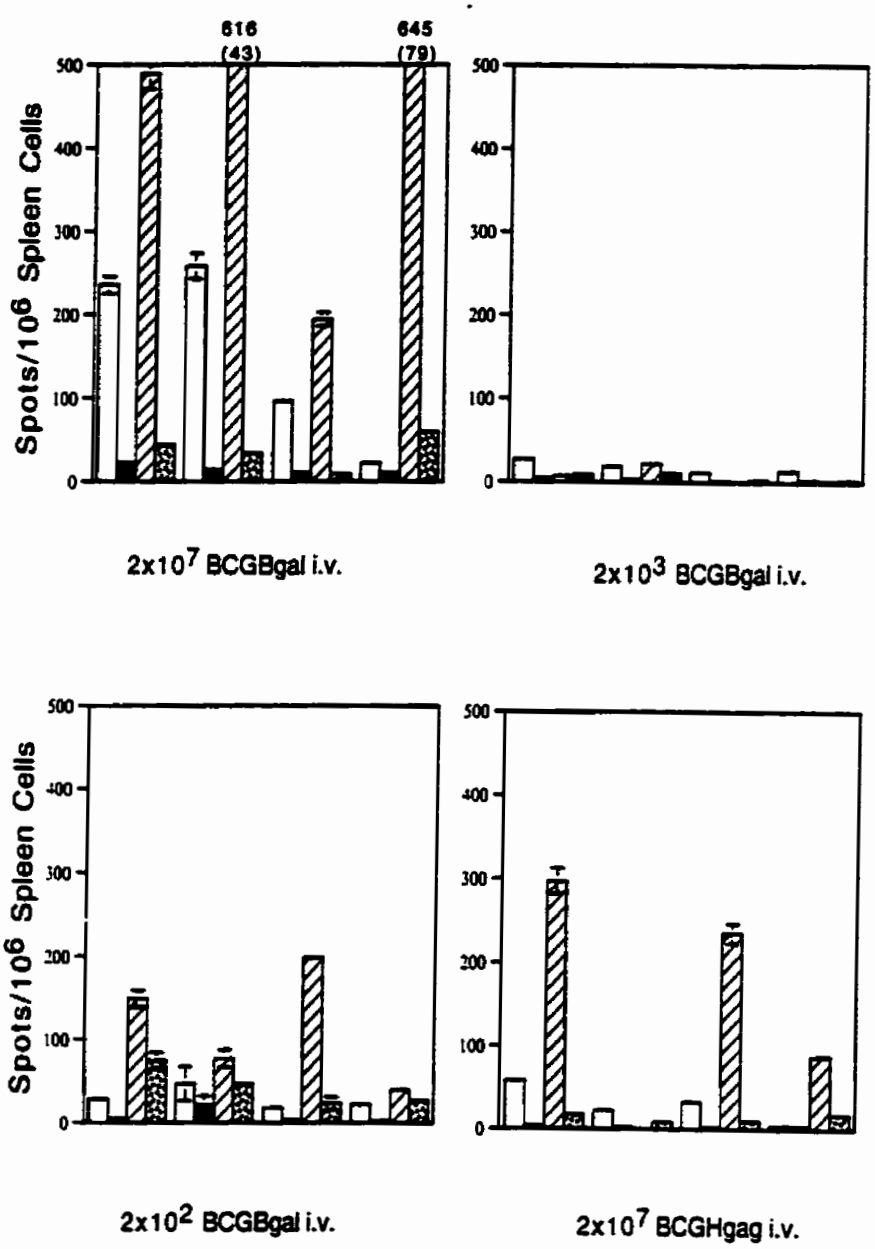



**Figure 5-13.** Immune response of individual mice vaccinated i.v. with different doses of rBCG after subsequent i.d. challenge with a high dose of rBCGβgal. Each group of mice was initially given an i.v. vaccination dose of a rBCG strain as indicated under each graph. After 4 months, the mice were challenged with a approximately 2 x$10^7$ rBCGβgal cfu given i.d. After an additional 16 wk, the mice were killed and the number of β-gal- and BCG-specific IFNγ- and IL-4-producing cells in their spleens was determined by ELISPOT assay. Open bars - IFNγ response to BCG; Black bars - IFNγ response to β-gal; Hatched bars - IL-4 response to BCG; Stippled bars - IL-4 response to β-gal.

imprinting in this group was unsuccessful. The response from mice that were immunized with $2x10^3$ rBCGBgal i.v. and then challenged with $2x10^7$ rBCGβgal are strikingly different. These mice appear to have a very small T cell response to both antigens, a response quite different from the control animals that were similarly challenged. The reason for this is unknown. Perhaps at this dose, these mice developed an effective immune response and thus, were able to clear the challenge dose rapidly, leaving few remaining effector T cells at the time of testing, i.e., 16 wk instead of ten.

The anti-β-gal serum antibody titers from the mice of Figures 5-12 and 5-13 are shown in Figures 5-14 and 5-15 respectively. Note the prominence of IgG2a antibody in the serum of mice immunized with a low dose of rBCG before challenge, compared to the control.

I wished to compare the immune response to β-gal in mice immunized with rBCGβgal and in mice immunized with purified β-gal by other methods that would be acceptable for immunization of humans. Figure 5-16 shows the immune response in mice immunized with purified β-gal given as a soluble protein in saline or in alum as an adjuvant, the only adjuvant approved for use in humans. In both cases, immunization was by the i.p. route. Soluble β-gal did not induce a significant immune response in any of the mice immunized with this preparation. β-gal in alum, however, induced a predominantly Th2 response in mice at even the low dose used. This is not unexpected as alum is particularly effective at inducing Th2 responses. However, these results exemplify potential problems in achieving a Th1 response in humans with current vaccination procedures.

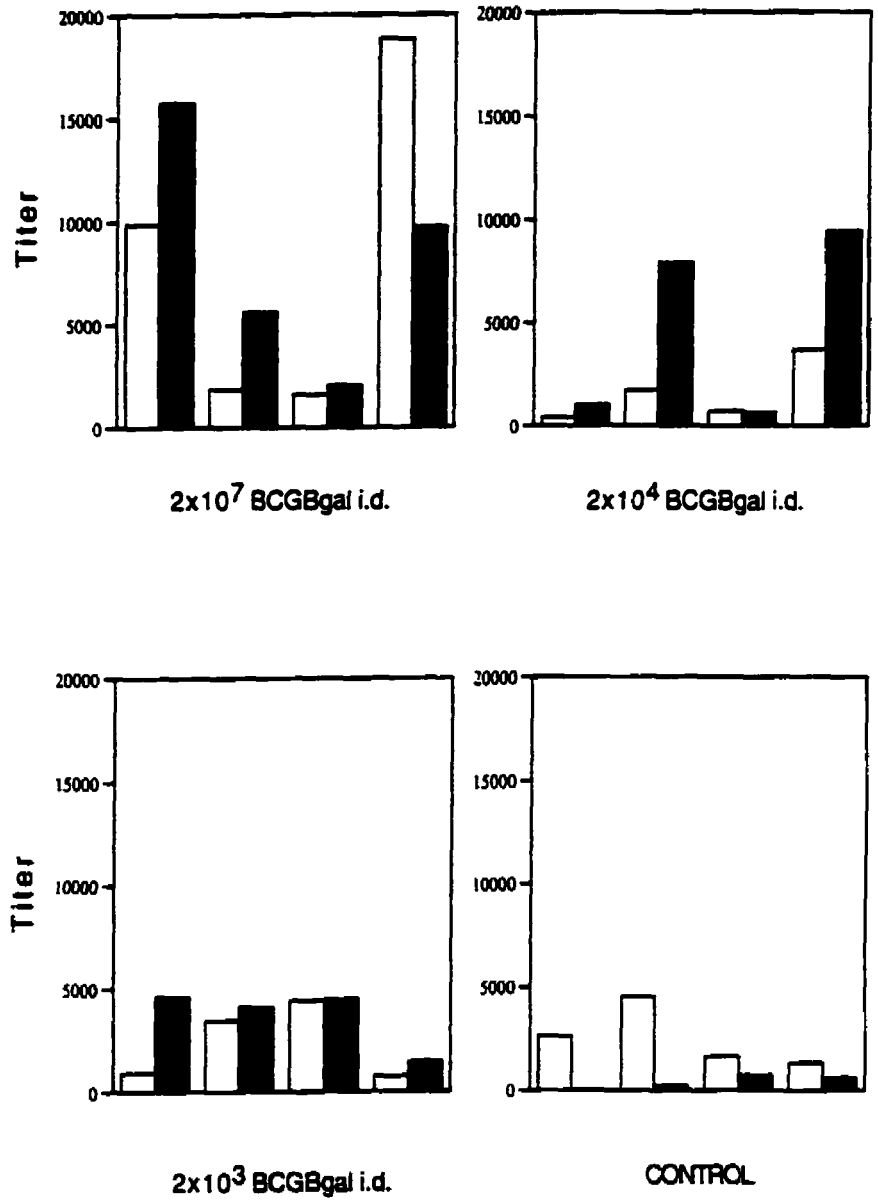



**Figure 5-14.** Serum antibody responses of mice to β-gal after immunization with different doses of rBCG by the i.d. route, and subsequent challenge with a high dose of rBCG by the same route. The results for unimmunized, age-matched control mice are also shown. Mice were immunized initially with the dose of rBCG indicated below each graph. After 16 wk., all groups were challenged with $2x10^7$ rBCG given intradermally. Serum was collected at the time the mice were killed, 16 wk after the challenge. Open bars show IgG1 titers while black bars depict IgG2a titers.

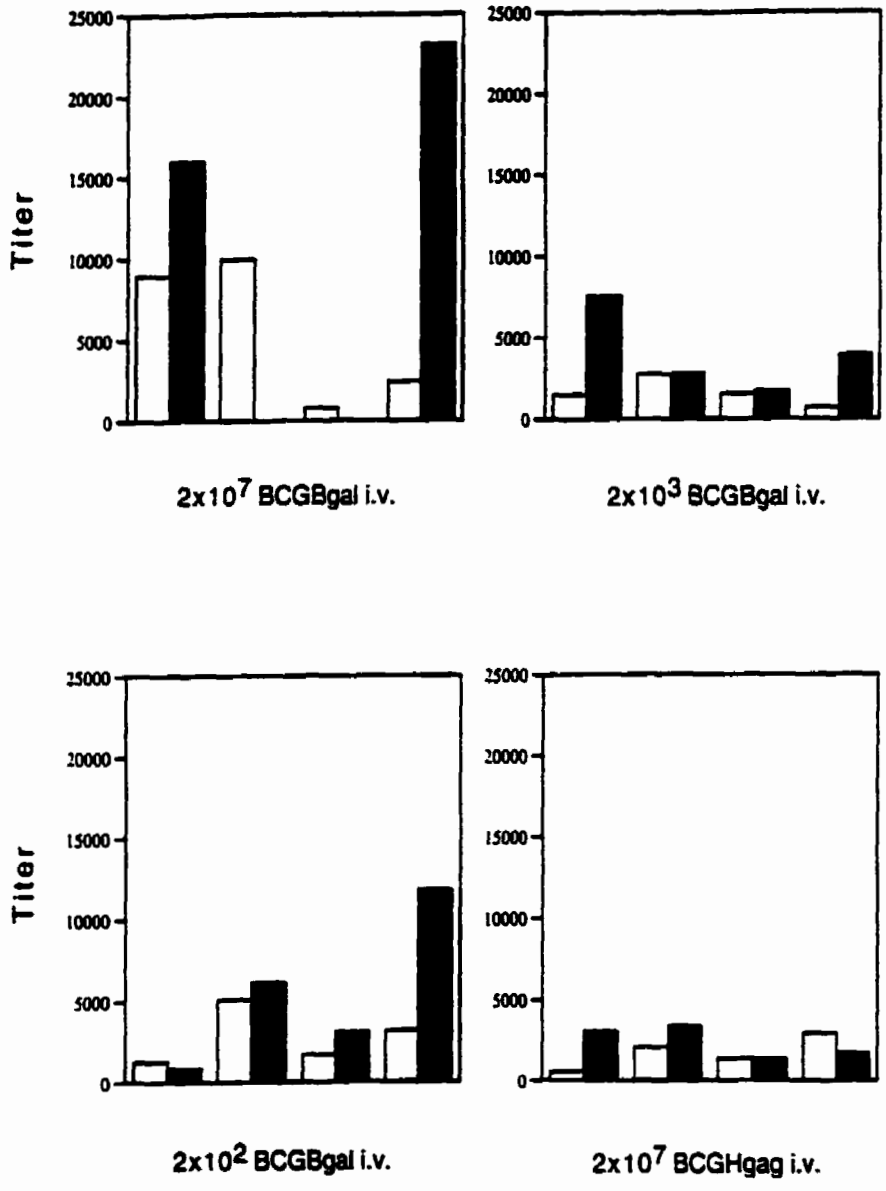



**Figure 5-15.** Serum antibody responses of mice to β-gal after immunization with different doses of rBCG by the i.v. route, and subsequent challenge with a high dose of rBCG by the i.d. route. Results for mice immunized with a high dose of BCG transformed with a control vector are also shown. Mice were immunized initially with the dose of rBCG indicated below each graph. After 16 wk, all groups were challenged with $2x10^7$ rBCG given intradermally. Serum was collected at the time the mice were killed, 16 wk after the challenge. Open bars show IgG1 titers while black bars depict IgG2a titers. See Figure 5-14 for the response of normal mice given only the challenge dose (Control).

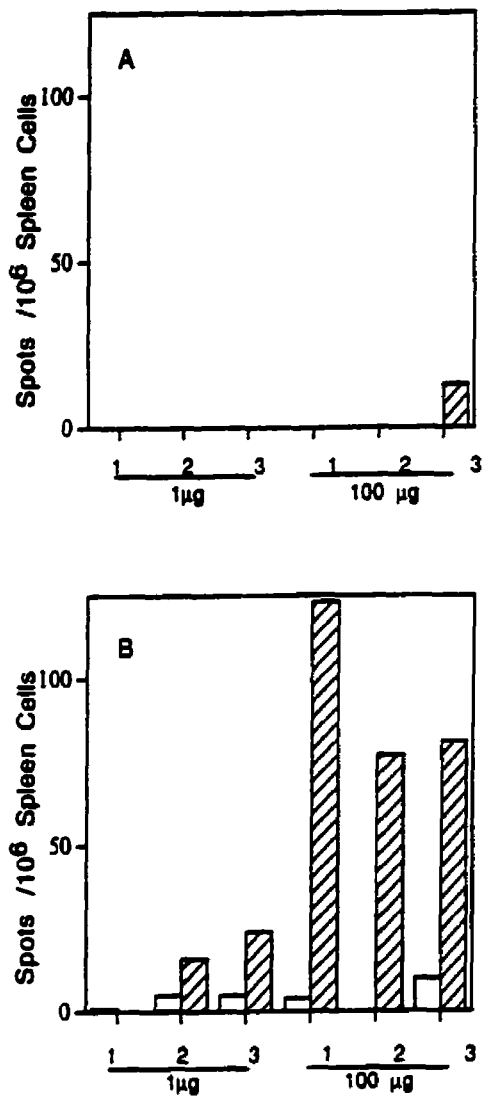



**Figure 5-16.** Number of IFNγ- and IL-4-producing cells in mice immunized intraperitoneally with soluble β-gal (A) and β-gal adsorbed to alum as an adjuvant (B). Soluble β-gal was administered twice five days apart, while β-gal/alum was administered only once. Mice were killed 11 days after the initial injection, and antigen-specific cytokine-producing spleen cells were enumerated by ELISPOT assay. Open bars represent IFNγ spots and hatched bars represent IL-4 spots.

## 5.3 Discussion

These results demonstrate that β-gal is expressed at significant levels after transformation of the BCG Montreal strain with pMV361::βgal. Expression of β-gal is much higher than that initially reported by Stover et al. (1991), who developed this plasmid and initially expressed β-gal. No doubt this is due to BCG strain differences as has been reported previously (Burlein et al., 1994). I have not estimated the in vivo expression of β-gal after injection; however, one study found that the activity of the hsp60 promoter in intracellular mycobacteria growing in cultured macrophages was similar to that of bacteria grown in broth culture (Dellagostin et al., 1995).

The dramatic effect that a simple variation in dose can have on the immune response to BCG is evident. I have shown, as have others, that rBCG is an efficient method for delivering antigen to mice. Not only do these results demonstrate a significant immune response to the recombinant protein, but unlike previous studies, they show that an almost purely Th1 response can be generated to the recombinant antigen by giving the rBCG at low doses. This is a very effective method for achieving this type of response in mice. Compare the immune response in mice immunized with soluble β-gal, either in saline or adsorbed to alum, with that in mice immunized with low doses of rBCGβgal. Perhaps not unexpectedly, the mice did not mount an immune response when given the soluble protein in saline, and they mounted a Th2 dominated response when given the alum preparation. Assuming that vaccination with the recombinant BCG is able to induce a similar pattern of response in humans, low dose vaccination with rBCG will be a very efficient mechanism for achieving effective vaccination for diseases for which a Th1 response is desired.

There remain many diseases for which an effective vaccine is required. Some of these diseases remain a problem because cell-mediated immunity is required to contain

infection, and vaccines that induce stable and effective CMI have not been established. The use of rBCG as a vaccine vector for immunizing against such diseases has been pursued for some time. However, the use of low doses of rBCG to ensure a Th1 response to the recombinant protein and subsequently upon exposure to a pathogen is novel and offers promise as a strategy to vaccinate against this type of organism. Three pathogens for which induction of a cell-mediated immune response by a low dose of rBCG offers promise are influenza, HIV, and Leishmania.

In consultation with my supervisor, Dr. Qualtiere, I decided that the influenza virus would be an appropriate model to assess the protective efficacy of low dose rBCG vaccination. The relative roles which antibody and cell-mediated immunity play in immunity to viruses are dependent on the specific virus. It is generally believed that antibody is more important for protection against cytopathic viruses while cytotoxic T cells are required for immunity to non-cytopathic viruses (Zinkernagel et al., 1996). In influenza virus infections, antibody is effective at limiting viral infection and in preventing reinfection with a homologous virus, but not against viruses with heterologous surface glycoproteins, hemagglutinitn (HA) and neuraminidase (NA). The internal structural proteins of influenza, NP and matrix protein (MA) are highly conserved among viruses of the same type but due to their location within the virus membrane, antibodies against these proteins have no effect in viral clearance. The role of cell-mediated immunity and in particular CTLs in protection against and clearance of influenza infections is poorly characterized. It is unclear whether a purely CMI response to this cytopathic virus will limit infection to a virus challenge. The most convincing evidence that cell-mediated immunity to influenza nucleoprotein is able to control an influenza infection comes from a paper by Ulmer et al. (1993). In experiments described in this paper, immunization of BALB/c mice with an influenza nucleoprotein DNA vaccine resulted in generation of both antibodies and CTLs. Mice immunized in this way were protected against normally lethal challenge with a heterologous virus. Furthermore, virus titers from lungs of immunized

mice challenged with a sublethal dose of virus were dramatically lower than those of unimmunized mice. Transfer of antibodies from immunized to unimmunized mice could not provide any protection from heterotypic challenge in recipient mice. Subsequently, other studies have shown similar results (Bot et al., 1996; Fu et al., 1997). These results indicate that, at least under some conditions, CMI to conserved influenza proteins can provide protection against heterotypic virus challenge. Thus, antibodies recognizing conserved surface epitopes can provide protection against homotypic influenza challenge, but CTLs recognizing internal viral proteins can also provide protection from heterotypic viruses.

However, infection with influenza virus does not always provide protection against viruses with antigenically different surface glycoproteins, even though they have nearly identical nucleoproteins and matrix proteins. I believe that this is due to the nature of the immune response that is generated upon exposure to influenza virus. If cell-mediated immunity but not antibodies can provide heterotypic immunity to influenza infection, then conditions of immunization that promote cell-mediated immunity will be protective against heterotypic challenge, while those that induce an antibody response will be ineffective. Th1 cells are more efficient inducers of CTL responses Than Th2 cells and furthermore, Th2 cells inhibit the promotion of CTL activity by Th1 cells (Palladino et al., 1991). The effect is likely mediated by IL-4 as this cytokine has been shown to reduce viral clearance in influenza infected mice when produced locally in the lung in transgenic mice (Bot et al., 2000) or after i.v. injection (Moran et al., 1996). Th1 cells, on the other hand, are potent inducers of CTL. In mice, the Th1/Th2 nature of the immune response to influenza virus depends on a number of parameters including the strain of mouse used (Graham et al., 1998); the age of the mouse at the time of exposure (Bot et al., 1997) and the type of antigen exposure, i.e., live virus, inactivated virus or subunit vaccines. It has been suggested that live virus infection induces a preferentially Th1 response in mice while, inactivated virus, which is currently used as the human

vaccine is thought to be more efficient for inducing a Th2 response (Moran et al., 1999). This may be true under standard conditions employed. However, direct comparisons between live and inactivated virus have not been made using a range of doses and similar routes of administration.

I propose that, in those who become ill after infection with influenza, a response with a Th2 component that is predominantly directed against the surface proteins HA and NA, results in induction of a humoral immune response to these proteins. This Th2 response inhibits the Th1 response to the virus and deviates the immune response away from the internal proteins. I hypothesize that this occurs for several reasons. The first is related to the replication of the virus, influenza being a very rapidly replicating cytopathic virus. The results presented here and the work of others (Lagrange et al., 1974; Parish, 1972) have demonstrated that antigen dose has a dramatic effect on the outcome of an encounter with antigen. I postulate that the high antigen load that the rapidly replicating influenza virus puts on the immune system results in immune deviation away from Th1, cell-mediated immunity towards a Th2, humoral response. The immunodominant surface proteins on the intact virus are also very efficient at stimulating B cells through the B cell receptor. The resulting humoral response is effective in neutralizing the virus. However, the antibody response to the surface glycoproteins is probably induced at the expense of the CTL response to internal proteins. Cytokines produced during a strong Th2 response have been shown to counteract the Th1 cytokines (Lehn et al., 1989; Sher et al., 1992). Studies of the CTL response to influenza in natural infection have identified CTLs specific for a number of epitopes of influenza proteins after infection with influenza, many of which are specific for NP. However, detection of cytolytic activity by these CTLs requires in vitro restimulation with antigen (Yewdell and Hackett, 1989). This suggests that although NP-specific CTLs are present after infection, their numbers or activity may not be at a significant level. Other studies have shown that Th clones specific for NP help in an in vivo antibody response to HA, but not for NP when T cell-deficient nude mice

are immunized with intact influenza virus (Scherle and Gerhard, 1986). Similarly, T cells primed to internal components of influenza virus by immunization with influenza virus particles from which the HA and NA spike proteins have been removed, are able to help in the antibody response to HA (Russell and Liew, 1979) when mice are immunized with intact virus. This is because these proteins are on the surface of the virus, allowing them to bind to B cell receptors and activate HA-specific B cells. Once the virus is internalized and degraded, NP epitopes presented on MHC-class II-molecules of the B cell, allow NP-specific Th cells to provide help to the HA-specific B cell. Internal proteins of intact viruses are unable to bind B cell receptors. Thus, the immune response is deviated not only towards Th2, but also towards the dominant HA and NA surface glycoproteins.

The manner in which the immune system responds on first contact with the virus sets the stage for future encounters with heterotypic influenza viruses due to the phenomenon of "original antigenic sin" (Fazekas-de-St.-Groth and Webster, 1966). Upon secondary exposure to the virus, the same type of immune response will be generated as in the primary infection due to the recall response to conserved regions in the surface glycoproteins. Evidence suggests that this phenomenon may also function in the CTL response to viruses as well (Klenerman and Zinkernagel, 1998). I propose that a cell-mediated immune response to internal virus proteins such as NP, is capable of providing protection against heterotypic viruses. Due to the dominance of the Th2 response to HA and NA both in natural infection and in vaccination responses, the Th1 and CTL memory responses are not strong enough to provide protection to surface glycoprotein variant viruses. I reason, therefore, that an immunization protocol that induces a Th1 immune response to conserved influenza proteins would be effective in providing heterotypic immunity to influenza. I believe that a recombinant BCG virus could accomplish this end.

An effective HIV vaccine has yet to be developed. This virus, like influenza, has variant surface glycoproteins that mutate over time, and this diversity contributes to the ability of the virus to escape the neutralizing effects of antibody. Unlike influenza, the virus can live for many years within an infected host accumulating mutations within an individual rather than a population, allowing the virus to escape successive waves of immune response. Vaccines that induce a humoral response to HIV are likely to be unsuccessful, because in order to prevent an infection, they must provide sterilizing immunity at the site of infection, an immune state that is very difficult to achieve.

There is much evidence to support a strong role for cell-mediated responses in providing protection against HIV (Cao et al., 1995; Rowland-Jones and McMichael, 1995; Fowke et al., 1996). As in the case of influenza, a rBCG expressing conserved HIV proteins may achieve this end (Salk et al., 1993).

The importance of the cell-mediated immune response in leishmania infections has been established (Locksley and Scott, 1991; Locksley et al., 1999). BALB/c mice are susceptible to leishmania infections with high numbers of parasites because they generate a Th2 response to this organism instead of the protective Th1 response. CBA mice are resistant to infection because they generate a Th1 response even at higher doses. However, when BALB/c mice are given a low dose of live leishmania parasites, they generate a protective Th1 response and are subsequently immune to further infections with much higher doses of the parasite (Bretscher et al., 1992; Menon and Bretscher, 1998). These experiments demonstrate the exquisite effectiveness of the low dose immunization strategy in another model system.

However, vaccination of humans with live leishmania parasites is not a practicality, and a safer method that achieves similar results must be developed for vaccinating people. Low dose vaccination with rBCG that express leishmania proteins is a promising alternative. In one experiment, BALB/c and CBA mice were immunized with BCG expressing leishmania gp63, and then challenged with *L. Major* promastigotes

(Connell et al., 1993). CBA mice spontaneously heal lesions, but some additional protection was provided by the rBCG vaccination as lesions in these mice were smaller and healed faster than in control mice. BALB/c mice were not protected from challenge by the *L. major* promastigotes. I predict that low dose vaccination with the recombinant BCGgp63 would be much more effective in inducing immunity to leishmania in BALB/c mice than the dose that was used in this study, just as a low number of leishmania parasites provides protection where a high dose does not. I believe that BCG expressing leishmania parasite-antigens will be a safe and effective alternative to low dose vaccination with the parasite itself.

# 6 Cloning and Expression of Influenza Nucleoprotein in *E. coli* and BCG

## 6.1 Introduction

In an effort to determine if low dose vaccination with rBCG expressing the influenza nucleoprotein could protect mice from subsequent infection with influenza virus, I attempted to clone the NP gene from influenza strain A/PR/8/34 and express it in BCG using the pMV361 plasmid. My intention of was to examine the immune response as well as the protective effect of NP expressed as a cytoplasmic protein in BCG. The plasmid pMV361 was chosen because it is a plasmid which integrates into the mycobacterial genome, thereby ensuring stable expression of the foreign gene.

## 6.2 Results

The gene was successfully cloned from purified viral RNA and sequenced. Figure 6-1 shows the amino acid sequence of the NP protein as predicted from the cDNA sequence. By comparison with the original published sequence of this strain (Winter and Fields, 1981), five nucleotide differences existed between the two sequences. Only three of these resulted in amino acid substitutions as indicated in Figure 6-1. Cloning of the gene into an *E. coli* expression vector pTrcHis enabled expression of recombinant NP as a fusion protein with a His•Tag in *E. coli*. Figure 6-2 shows lysate of *E. coli* transformed with pTrcHis::NP run on immunoblot and stained with a monoclonal antibody which recognizes the His•Tag. This figure shows that the full-length protein is

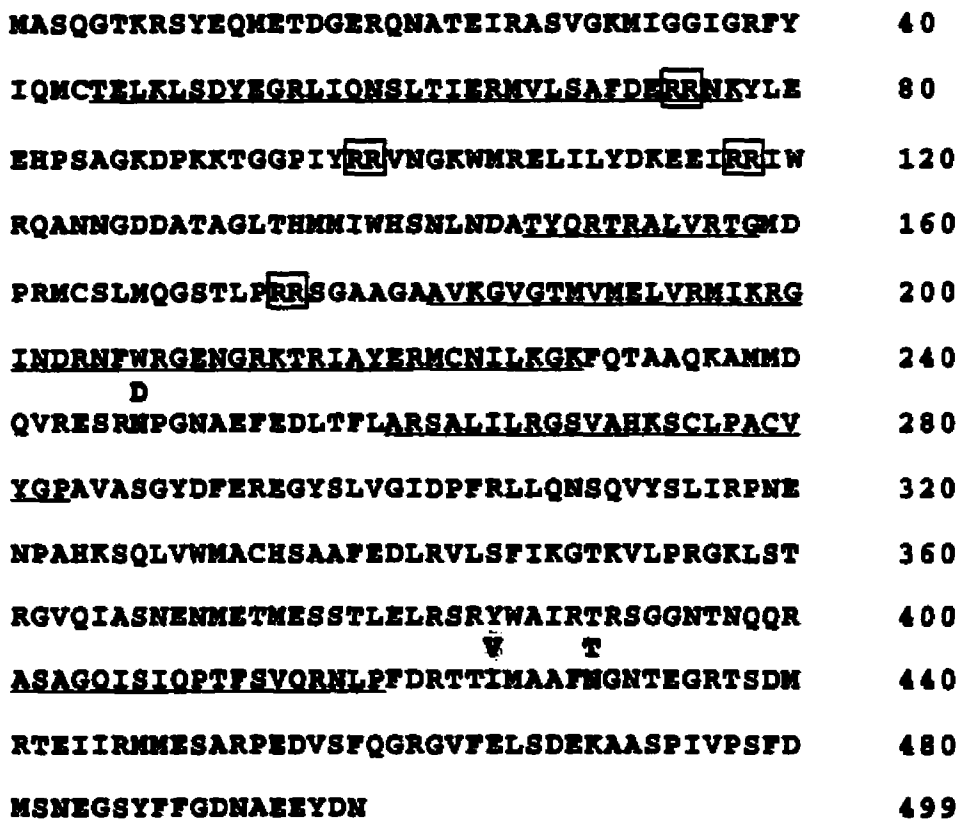



**Figure 6-1.** Amino acid sequence of the cloned influenza nucleoprotein. Shaded boxes indicate the amino acid differences between the original published sequence (Winter and Fields, 1981) and the newly cloned gene. Open boxes indicate the position of adjacent arginine pairs. Underlined areas are regions containing T cell epitopes for BALB/c mice (Brett et al., 1991).

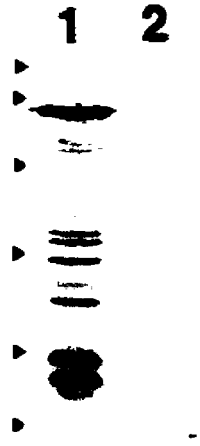
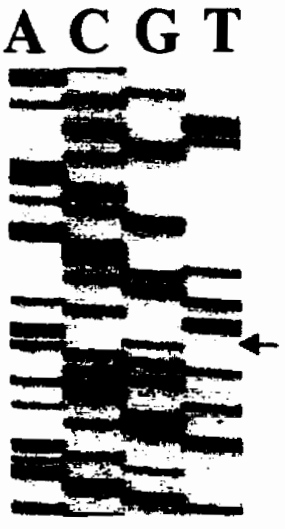



**Figure 6-2.** Immunoblot of *E. coli* lysate stained with a monoclonal antibody that recognizes the His•Tag on the NP protein. Lane 1 contains lysate from *E. coli* transformed with pTrcHis::NP. Lane 2 contains lysate of *E. coli* transformed with the parent plasmid alone. The full length product is visible in lane 1; however, many other smaller protein products are also stained. Molecular weight markers are indicated by the arrowheads at the left, from top to bottom: phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (18.4 kDa), lysozyme (12.5 kDa).

produced, but that many other smaller products are also stained with the anti- His•Tag monoclonal antibody. No bands are visible in immunoblot of lysate from untransformed bacteria (Fig. 6-2), suggesting that the smaller protein products are truncated versions of the NP protein. Analysis of the cDNA sequence of the virus shown in Figure 6-1 reveals a series of arginine residues throughout the sequence. In particular, five pairs of adjacent arginines are located within the first 200 amino acids of the gene. Analysis of codon usage in *E. coli* indicates that transfer RNAs (tRNAs) for arginine are rare and, therefore, these arginine pairs (and perhaps other rare codons) may be responsible for ribosome stalling resulting in incomplete protein products (Hormeache and Khan, 1996). Of course, another explanation for the truncated NP proteins is that they are the result of proteolysis. However, preparation of the lysate in conditions designed to limit proteolytic cleavage, i.e., in the presence of protease inhibitors and at low temperature, did not change the banding pattern of the product, suggesting that proteolytic cleavage is not responsible for the multiple bands observed. Because the codons for arginine used in this gene require tRNAs that are rare in mycobacteria as well (Andersson and Sharp, 1996), this could present a problem for expression of the NP gene in BCG also.

The NP gene was cloned into the mycobacterial expression vectors pMV261 and pMV361. Figure 6-3 shows the sequence of the junction between the Eco RI restriction site in the pMV361 vector and the NP gene fragment. The fragment is in frame, but expression of NP from BCG transformed with expression vectors containing the NP gene proved difficult. BCG transformed with pMV261::NP did not form colonies suggesting that the NP protein may be toxic to the cells. Similar results have been found for other proteins expressed in BCG. Colonies were formed when BCG was transformed with the genome integrating vector pMV361::NP; however, no significant expression of the gene could be detected either by immunoblot of the rBCG lysate or by an immune response to NP in mice after immunization with a high dose of the rBCG. Thus, it seems that the NP protein is toxic to BCG and that the transformed organisms can only grow when the protein is not expressed, or expressed at very low levels.

A.




B.

ATG GCC AAG ACA ATT GCG GAT CCA
GCT GCA GAA TTC ATG GCG TCC CAA
GGC ACC AAA CGG TCT TAC GAA . . . .

**Figure 6-3.** Sequence of the junction between the pMV361 plasmid and the NP gene. A. Sequencing gel showing the junction between the plasmid and the NP gene insert at the Eco RI restriction site (arrow). B. The NP gene sequence is in frame with the ATG start of the first six amino acids of the Hsp60. The Eco RI site is underlined.

## 6.3 Discussion

These findings put some limitations on the ability to use low dose rBCG to vaccinate against influenza. Firstly, it appears that NP is not expressed efficiently in BCG, at least from the vector of choice, pMV361. This may be due in part to codon usage. As in *E. coli*, tRNAs recognizing the codons for many of the arginine residues are rare in mycobacteria (Andersson and Sharp, 1996). This problem could be alleviated by site-directed mutagenesis to change the offending codons. However, this would simply cause another problem since the expressed protein appears to be toxic to the mycobacteria. To eliminate the toxicity problem, there are several approaches one could take. First, by creating deletion mutants of the NP protein, one could try to determine the areas that are toxic to the bacteria. Analysis of the functional domains of the protein may have some predictive value in determining these (Kobayashi et al., 1994). The influenza NP binds to influenza RNA gene segments without sequence specificity (Baudin et al., 1994). It is likely that the NP protein, when expressed in mycobacteria, will bind to the bacterial RNA inhibiting normal function within the cell. Thus, eliminating portions of the gene coding for RNA binding portions of the protein might prevent the problem of toxicity. However, the areas of the NP protein which are responsible for RNA binding, the first 200 amino acids (Kobayashi et al., 1994), are also immunologically important areas (see Figure 6-1). In particular, for BALB/c mice, which are H-$2^d$, the only class I-restricted epitope lies within this area (Brett et al., 1991). Eliminating immunologically important areas could significantly alter the ability of the immune system to respond.

Certainly for human vaccination purposes, it is preferable to have a recombinant vaccine that contains the entire NP sequence, as this will have the greatest chance of containing epitopes recognized by T cells of all members of the population. This is what I attempted to do initially, but it may not be possible. For experimental purposes, it may be more practical to choose certain epitopes of immunological importance to a particular mouse strain such as BALB/c, and to express these peptides as a fusion protein with a

protein that is well tolerated and expressed at reasonable levels by BCG. This would allow the testing of various hypotheses of the low dose rBCG vaccination strategy, presumably without the complications of dealing with the intact protein. If this work is to continue, I suggest that this is the next logical step. However, for practical purposes of vaccinating humans, it would be interesting to explore the use of BCG vectors encoding a truncated form of NP, perhaps in combination with other conserved influenza proteins such as the matrix protein.

# 7 General Discussion

## 7.1 Implications of the Experimental Observations for Immune Class Regulation

### 7.1.1 Dose as a Determinant of Immune Class

The results presented here should be of interest to those studying basic immunological mechanisms in several respects. First of all, as in previous studies from this laboratory (Bretscher et al., 1992; Menon and Bretscher, 1996; Menon and Bretscher, 1998), they demonstrate important characteristics of immune regulation that are not commonly recognized. The immune system seems to follow some very simple rules in responding to antigen. These simple rules dominate decision making by the immune system and it seems that many other variables, which have been determined to be important in immune class regulation, may be at least partially secondary to antigen dose. The fact that the immune system can respond to complex and simple antigens in similar ways, and that merely changing the dose of antigen administered can dramatically change the immunological outcome of antigen exposure, suggests that the physiochemical nature of the antigen is of less importance than the dose. In numerous vaccination studies using antigens of varied levels of complexity, from simple proteins to complex pathogens (Asherson and Stone, 1962; Parish, 1972; Bretscher et al., 1992; Bancroft et al., 1994; Menacci et al., 1996; Menon and Bretscher, 1998; Power et al., 1998), dose has proven to be the determining factor in the type of immune response generated, with low doses inducing predominantly type 1 responses and higher doses inducing either type 2 responses or mixed type 1/type 2 responses. A low dose of a protein antigen induces a DTH response, while higher doses result in antibody production (Parish, 1972).

Similarly, administration of relatively low numbers of XRBC (Ismail and Bretscher, 1999; Lagrange et al., 1974), heat-killed *Mycobacterium vaccae* (Rook et al., 1981) or slowly replicating viable organisms such as BCG mycobacteria (this work and Power et al., 1998), or leishmania parasites (Menon and Bretscher, 1996; Menon and Bretscher, 1998) initiates a Th1 response while higher numbers induce either a mixed Th1/Th2 or predominantly Th2 response. Thus, the nature and complexity of the antigen are subordinate to the dose of antigen administered in determining the class of immune response generated. This is not to say that the physiochemical nature of antigens is not an important factor in immune class regulation. This is evidenced by the fact that although low doses of both BCG and leishmania induce Th1 responses, high numbers of leishmania parasites induce almost exclusive Th2 response while high numbers of mycobacteria induce a mixed response. This is most likely due to the presence of many immunomodulatory molecules within the mycobacteria that exert their effects more strongly at high doses. However, the simple fact that low doses of many antigens can almost always induce a Th1 response suggests that dose is dominant over the nature of the antigen.

Similarly, dose of antigen appears to be dominant over the route of exposure as a factor in determining the class of immunity that is generated in an immune response. In the various routes that have been assayed, i.v., s.c. and i.d., the type of response generated could be controlled simply by varying the dose of antigen, suggesting that the low dose strategy works independently of the route of administration. However, among the various routes, the antigen dose range differed. With i.v. administration of BCG, a low dose was in the range of $10^2$-$10^3$, while for the i.d. route it was at least ten fold higher. This is not unexpected as previous work has demonstrated that antigens are more effective immunogens when administered by some routes than by others (Lagrange et al., 1974), most likely because the antigens have greater access to secondary lymphoid organs. None the less, in all experiments reported here, a relatively low dose produced a

predominant Th1 response and a relatively high dose induce a mixed Th1/Th2 response. This observation highlights the importance of looking at the response to a full range of doses before discussing the ability of a particular route of immunization to induce a particular type of response.

It must be noted that low dose vaccination to achieve a stable Th1 imprint works only with non-replicating antigens when administered repeatedly over a number of weeks, or with an infection of a very slowly replicating organism. This is because it takes time for the stable immune response to develop, and chronic, low level stimulation of the immune system is required during this time to prevent the development of a Th2 response.

Why should the immune system respond to low doses of antigen with the generation of a relatively exclusive Th1 response? Consider that the immune system of mammals has co-evolved with various infectious pathogens. The immune system must have evolved not only mechanisms to deal with the various diverse pathogens, but also an ability to decide which mechanism is appropriate for dealing with each organism it encounters. As it is impossible for a species to develop a mechanism for dealing with each particular pathogen (particularly before encountering it), the immune system must have developed a set of general rules to allow it to respond to the largest number of potential pathogens most effectively. In order to do this, the immune system must take its cues from the infecting organism in determining how it will respond. These cues must be general properties shared by large numbers of organisms that provide the immune system with clues about how to initiate an effective response. The clues are likely to be simple characteristics of the pathogen such as the site of infection, the site of replication of the organism (intracellular vs. extracellular), as well as the rate of replication of the organism.

In some sites, certain classes of immunity dominate the response, i.e., at mucosal sites, immunoglobulin-$\alpha$ (IgA) production by B cells is strongly favoured. Thus, any antigen that is presented at a mucosal surface favours an IgA response regardless of the

nature of the antigen. This makes sense because, regardless of the nature of the antigen, binding of IgA will allow it to be neutralized without the generation of a more damaging inflammatory response. None the less, even in the gastrointestinal tract, dose of antigen has a role to play in determining the Th1/Th2 nature of the immune response (Bancroft et al., 1994).

Similarly, the location where a pathogen resides in vivo has a significant influence on the type of immune response it induces. Intracellular pathogens are much more effective at inducing MHC-class I-restricted T cells than those that reside extracellularly. Protein antigens which are introduced into this compartment are also effective inducers of class I-restricted T cells (Moore et al., 1988). Thus, it is a characteristic of the organism, the location in which it resides in vivo, which determines the efficiency of induction of this class of immunity.

I believe that the immune system has evolved mechanisms for determining which type of T helper response is appropriate for a given infection, and that the decision is based, to a large extent, on the amount of antigen which is "perceived" by the responding immune cells. There is evidence to suggest that immune responses follow a particular pattern, with Th1/cell-mediated immunity being induced first, followed thereafter by the Th2/humoral response (Salvin, 1958). However, in many cases an exclusive Th1/cell-mediated immune response is induced. I believe that the signal that tells the immune system to mount a Th2 response is a higher antigen load. There is a logical explanation as to why this should occur. In an initial infection, low antigen dose induces the initial Th1, cell-mediated response. If the infecting organism is a slow-growing organism, then the antigen load remains low and the immune response remains a cell-mediated one. Cell-mediated responses can be particularly effective for eliminating infecting organisms, but in the process, they can be very destructive to host tissues. When the number of infecting organisms is small, the body can handle a limited amount of tissue destruction in favour of eliminating the infection, a small sacrifice to ensure that the infection is contained.

However, with an increased pathogen load, the tissue damage caused by a cell-mediated response becomes a much more serious problem, as the damage caused by the immune response may be detrimental or lethal to the host, a sacrifice too great for the benefits. Thus, at higher antigen loads, a humoral response which is less destructive to host tissues would be preferable.

Why should such destructive cell-mediated mechanisms exist if the humoral responses can eliminate an infection? The answer is related to the functional ability of the two different arms of the immune system. Cell-mediated responses can function at very low levels of antigen stimulation. A T cell can be activated to perform some functions at extremely low levels of T cell-receptor (TCR) occupancy, while antibody-dependent complement-mediated cell lysis, for example, requires approximately $10^5$ IgG molecules bound to a cell in order to be effective (Humphrey and Dourmashkin, 1969). This number is required to ensure the likelihood that two of them will be sufficiently close to allow for cross-linking of, for example C1q, the initiating component of complement. Thus, at low antigen load, cell-mediated immunity would be a functional effector mechanism, while at higher antigen loads a humoral response would be operational and far less damaging.

### 7.1.2 Coherence

The experimental results presented here and elsewhere (Bretscher et al., 1992; Menon and Bretscher, 1998; Power et al., 1998; Ismail and Bretscher, 1999) also demonstrate another little considered characteristic of the immune system. The Th cell response to all epitopes physically associated with a discreet entity, be it a protein, a virus particle, a bacterium or parasite, will be regulated such that all responding cells will tend to have a similar helper phenotype. Such regulation is referred to as *coherence* (Bretscher, 1994) and I believe that it reflects the need to ensure that the host is able to mount a directed and effective immune response. If the immune system did not have such

a mechanism to coordinate the immune response, I would expect that the response to each individual protein or peptide of BCG would be different, i.e., there would be both Th1 cells and Th2 cells responding to each of the various epitopes in a protein and each of the proteins within the organism. This is not the case, as in mice immunized with a low dose of rBCG we see a predominantly, if not exclusive, Th1 response to the expressed foreign protein β-gal, indicating that a mechanism to maintain a coherent response to each epitope of an individual protein must exist. Furthermore, as shown in chapters 4 and 5, the immune response to all proteins that are physically linked as part of one entity, such as BCG, are coordinately regulated such that the Th cells responding to all individual epitopes of individual proteins tend to be of the same type.

There are rational arguments as to why such a mechanism should exist. Consider an individual who is infected with a complex organism such as *M. tuberculosis*. This intracellular organism can be controlled by cell-mediated immunity, but a humoral response is ineffectual in eliminating an infection. Furthermore, cytokines produced by Th2 cells may actually counteract the effect of the Th1 cytokines, thereby inhibiting the effect of the appropriate immune response. Studies have also shown that the Th2 cytokine, IL-4, alters the function of the Th1 cytokine TNF, making it more destructive to host tissues (Hernandez-Pando and Rook, 1994; Hernandez-Pando et al., 1997). Thus, it is imperative that the immune system is able to mount an appropriate type of response to all possible epitopes of an infecting organism to ensure efficient clearance of that organism with the least possible damage to the host. If such a mechanism did not exist, it is hard to imagine how a directed and effective immune response could be generated to any infectious organism. Understanding how coherence is maintained is critical to understanding immune regulation. One theory suggests that coherence is established through T cell-T cell cooperation, and experimental evidence supports this theory (Bretscher, 1994).

## 7.2 Factors that Influence T helper Responses

The class of immunity that emerges in an encounter with antigen can be influenced by numerous variables acting throughout the development of the immune response. As the class of immunity often determines the efficacy of the immune response, an understanding of immune class regulation is essential for effectual immune intervention in infectious diseases. This work demonstrates that mycobacterial dose is a significant factor in determining the Th1/Th2 nature of the subsequent immune response. Knowledge of other factors involved in the Th1/Th2 fate of cells, their mechanism of action and their relative importance at various stages of the immune response to pathogens, will provide a better understanding of disease pathology and will ensure effective disease interventions. The following sections discuss some of these variables.

### 7.2.1 Genetic Factors which Predispose Individuals to Develop Polarized Immune Responses

Inbred mouse strains demonstrate great variability in the type of immune response they generate upon exposure to leishmania parasites, an intracellular parasite that can only be controlled by a Th1 response. BALB/c mice develop a Th2 dominated response and are unable to control infection when infected with relatively high numbers of parasites and are considered a susceptible strain (reviewed in Locksley et al., 1999). Other strains are resistant to leishmania because they generate a Th1 response and control the infection when administered a similar number of parasites (Locksley et al., 1999). The genetic predisposition to develop polarized Th responses reflects the combined effects of multiple genetic loci rather than a single gene (Bix et al., 1998; Coffman and Beebe, 1998). Genes involved in the Th1/Th2 decision following leishmania infection in mice include MHC-genes (Roberts et al., 1997), and those affecting cytokine production (Bix et al., 1998) and the ability to respond to cytokine signals (Gorham et al., 1996). In humans,

susceptibility to mycobacterial infections has similar genetic associations (Altare et al., 1998b; Bothamley, 1999; Bothamley et al., 1989).

In the leishmania model, genetic susceptibility or resistance are not absolute traits since curing Th1- or non-curing Th2-dominated responses can be generated in both "susceptible" BALB/c mice and "resistant" CBA mice simply by varying the dose of parasite administered (Bretscher et al., 1992; Menon and Bretscher, 1996; Menon and Bretscher, 1998). Therefore, a genetically diverse mouse or human population demonstrates considerable variability in the Th1/Th2 nature of the response generated upon infection. However, it is probable that the large majority of individuals could mount either a Th1 or Th2 dominated response depending on the conditions of exposure to the organism.

### 7.2.2 The Cytokine Environment of T Cells

IL-12 is the main differentiating cytokine for Th1 cells (Abbas et al., 1996; O'Garra, 1998). Early exposure of activated Th cells to IL-12 affects the regulatory elements of the IFNγ promoter, resulting in increased transcription of the IFNγ gene ((Zhang et al., 1998). Thus IL-12 produced by antigen-presenting cells can significantly increase the production of IFNγ from T cells, and stimulate Th1 cell differentiation. However, IL-12 deficient mice are able to mount a Th1 type response to virus infections (Oxenius et al., 1999) and IFN-regulatory factor-1 gene-disrupted mice which do not produce either IL-12 or interleukin 18 (IL-18) can mount a Th1 response upon infection with plasmodium (Feng et al., 1999a). These studies suggest that while IL-12 is a potent inducer of Th1, it is not an absolute requirement for the development of the Th1 phenotype.

IL-4 is required for the differentiation of newly activated precursor T helper cells (pTh) cells to the stable IL-4-producing, Th2 phenotype (Abbas et al., 1996; Bix et al.,

1998; O'Garra, 1998). Consequently, several groups have sought a non-T cell source of IL-4 that could prime newly activated T cells to produce IL-4 (O'Garra, 1998). However, simply because IL-4 activity is required for development of cells with a stable Th2 phenotype does not mean that newly activated Th cells require IL-4 stimulation before they can produce IL-4. Indeed, the newly activated Th cell itself can be a source of IL-4 that acts in an autocrine manner to drive Th2 differentiation (Schmitz et al., 1994; Rincon et al., 1997; Bix et al., 1998; Coffman and von der Weid, 1999). Thus, IL-4 from an extraneous source is not obligatory for Th2 differentiation.

The mechanism by which IL-12 and IL-4 promote Th1 and Th2 responses, respectively, is as yet unclear. Whether such cytokines induce differentiation of pTh cells into polarized subsets or simply promote expansion of polarized clones is a topic receiving considerable attention (Coffman and Reiner, 1999; Reiner and Seder, 1999). It is probable that both mechanisms are involved.

There are several situations in which the cytokine environment is likely to play a significant role in the Th1/Th2 nature of the immune response.

i. Some pathogens, particularly bacteria, possess factors that directly stimulate cells to produce cytokines. These factors include bacterial cell wall components such as lipopolysaccharide (LPS) and mycobacterial lipoproteins (Brightbill et al., 1999; Watanabe et al., 1999), bacterial DNA CpG motifs (Bendigs et al., 1999; Cowdery et al., 1999), and toxins such as cholera toxin (Foss et al., 1999). LPS and a 19 kDa mycobacterial lipoprotein stimulate IL-12 production in macrophages by binding to Toll-like receptors on these cells (Brightbill et al., 1999). Microbial DNA containing CpG motifs is a potent inducer of inflammatory responses, inducing production of IL-12 by cells of the monocyte lineage (Cowdery et al., 1999). Recent results have demonstrated that these sequences can also have direct effects on T cells, inducing IFNγ (Iho et al., 1999), as well as IL-2 production and proliferation (Bendigs et al., 1999). These

immediate responses to pathogen components probably reflect employment by the immune system of an innate defense mechanism, and the early cytokine production could potentially influence the fate of Th cells.

ii. Conversely, many pathogens are able to escape immune detection through their ability to exert negative influences on the immune response. Macrophages infected with mycobacteria demonstrate reduced ability to respond to IFNγ-signaling (Hussain et al., 1999; Ting et al., 1999). Retroviral infection inhibits IL-12 production by mouse dendritic cells or human peripheral blood mononuclear cells (Kelleher et al., 1999; Marshall et al., 1999) and a similar function has been demonstrated for leishmania phosphoglycans (Piedrafita et al., 1999). In addition, LPS and its lipid A moiety have been shown to directly inhibit IL-4 production in T cells (Watanabe et al., 1999). In such cases, the absence of differentiating cytokines could act to sway the immune response away from a protective one.

iii. In an ongoing immune response or a secondary response to a pathogen, polarized effector Th cells (eTh) are likely to be present during the initial activation of pTh cells. Cytokines produced by these eTh cells could influence the fate of the newly activated cells maintaining a coherent response to the pathogen. Similarly, the lineage of APC involved in activating Th cells can influence their fate (Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Rissoan et al., 1999; Saha et al., 1999; Stumbles et al., 1998). Generation of these different dendritic cell subsets involves exposure to distinct cytokines during maturation, and results in cells with vastly different cytokine profiles (Kalinski et al., 1999; Maldonado-Lopez et al., 1999) and surface markers (Stumbles et al., 1998), which can, in turn, help to polarize Th cells.

iv. Infections with pathogens such as *Toxoplasma gondii* (Santiago et al., 1999) result in very potent cytokine responses. It is probable that the cytokines produced by these cells influence the fate of Th cells responding to non-cross-reacting antigens (see section 7.2.6).

### 7.2.3 Strength of Signal Model and Cell Cycle Control of Cytokine Expression

In many situations, the Th1/Th2 fate of cells is determined in the initial absence of exogenous polarizing cytokines such as IL-12, IFNγ and IL-4. A model that describes how the fate of Th cells is determined in such cases is necessary for developing a better understanding of immune class regulation. The "strength of signal" model suggests that higher TCR occupancy and costimulation are required to induce a Th2 response than a Th1 response. Many experimental observations support such a model (Bretscher et al., 1992; Corry et al., 1994; Brown et al., 1997; Power et al., 1998; Saha et al., 1998).

Recent studies by Reiner (Bird et al., 1998) and Hodgkin (Gett and Hodgkin, 1998) have suggested a mechanism by which cell cycle control of cytokine expression could determine Th1/Th2 differentiation. Upon activation of pTh cells, initial expression of cytokines varies depending on cell cycle and cell division (Bird et al., 1998; Gett and Hodgkin, 1998). Upon stimulation of naive $CD4^+$ cells, IL-2 is produced before the cell enters cell cycle, while IFNγ production requires progression of the cell into S phase. IL-4 is not produced until cells have gone through three or more rounds of replication even if IL-4 is present in the culture (Bird et al., 1998). It has been suggested that chromatin restructuring, necessary to allow for subsequent transcription of some cytokine genes, takes place during cell division (Bird et al., 1998; Reiner and Seder, 1999).

I espouse a model in which IL-4 production by T cells does not reflect a requirement for a definitive number of cell divisions, but rather a minimum rate of replication. For cells that do not meet this minimum rate of cell division, the default is a Th1 phenotype while those that divide at or above this minimum rate of division begin to produce IL-4. At low levels of stimulation (TCR occupancy, costimulation), the pTh cell is weakly stimulated and cell division progresses slowly. However, IL-2 and IFNγ are produced and IFNγ stimulates IL-12 production by antigen-presenting cells. At low levels of stimulation, the Th cell does not reach the critical rate of cell division required for IL-4 production. Meanwhile, autocrine IFNγ has a negative influence on the ability of the cell to produce IL-4, possibly through epigenetic modification of the IL-4 gene and/or though regulation of transcription factors (Lederer et al., 1996). Under conditions of high antigen stimulation, the Th cell divides rapidly, quickly acquiring the ability to produce IL-4, which acts in an autocrine fashion to stimulate Th2 cell differentiation.

### 7.2.4 The Dose and Nature of Antigen and Route of Infection

In support of the strength of signal model, the initial dose of a simple antigen or complex organism administered to an animal has a significant effect on the outcome of an immune response, as discussed throughout this thesis and in many publications on the topic. None the less, results of dose response studies which have used transgenic mice are often opposed to the results discussed above, with high doses of peptide mediating a Th1 response and low doses mediating Th2 responses (Grakoui et al., 1999; Rogers and Croft, 1999). Experiments involving non-transgenic mice and intact antigens at physiologic concentrations are more likely to reflect the situation observed in natural infections. However, the differences between these two models deserve explanation. An attempt has been made to reconcile these observed differences (Rogers and Croft, 1999).

### 7.2.5 Number of T cells Available to Respond

The number of Th cells available to respond to a particular antigen is a central factor in determining the fate of the responding Th cells (Mitchell et al., 1981; Powrie et al., 1994). BALB/c mice are susceptible to infection with leishmania when inoculated with a relatively high dose of the parasite. Nude mice reconstituted with large numbers of BALB/c lymphocytes are also susceptible. However, nude mice reconstituted with low numbers of BALB/c lymphocytes produce a healing Th1 response (Mitchell et al., 1981; Powrie et al., 1994). These results suggest that more responding Th cells are required for development of a Th2 response, whereas fewer Th cells favor development of Th1 cells. Similar results have been achieved using XRBC as an antigen (Ismail and Bretscher 1999). Reconstitution of irradiated mice with high numbers of syngeneic spleen cells results in a Th2 response upon immunization with a large inoculum of XRBC, while reconstitution with lower numbers results in a Th1 response. However, mice reconstituted with the higher number of lymphocytes developed Th1-dominated responses when immunized with a low number of XRBC (Ismail N and Bretscher PA, unpublished data). Thus, Th cell fate is determined by an interplay between the number of T cells available to respond and the amount of antigen to which they are responding. A model that explains how the number of responding T cells and the amount of antigen can conjointly regulate the Th1/Th2 fate of immune responses has been proposed (Bretscher, 1996).

### 7.2.6 Concurrent Infections

Mycobacteria are potent inducers of immune responses, having immunostimulatory properties, which promote Th1 responses in particular. Vaccination with *M. bovis* BCG has been shown to sway subsequent immune responses towards the Th1 pole. Schoolchildren who received BCG vaccination were less likely to develop atopic reactions than were those who had not been vaccinated (Shirakawa et al., 1997).

Similar results were observed in an animal model (Herz et al., 1998). Likewise, *Toxoplasma gondii* infection, which induces a Th1 response upon infection of BALB/c mice, can prevent the development of a Th2 response and progressive disease upon infection with *Leishmania major* (Santiago et al., 1999). Conversely, helminth infections which tend to induce potent type 2 immunity can divert Th1 response towards the Th2 pole (Brady et al., 1999; Malhotra et al., 1999; Stewart et al., 1999). These results suggest that a strong, ongoing systemic response can significantly influence the Th1/Th2 nature of the response to an unrelated antigen. None the less, it is possible to achieve independent immune responses to non-cross-reacting antigens in the same lymphoid organ (Ismail and Bretscher, 1999). Therefore, the influence of these infectious agents on immune responses to non-related antigens reflects pathological effects of an overwhelming infection rather than a normal regulatory mechanism of the immune system. The mechanism(s) by which these potent immune responses exert their effects on responses to unrelated antigens remains to be determined.

### 7.2.7 Concluding Remarks

The work presented in this thesis illustrates the effect of vaccination dose on the outcome of the immune response to mycobacteria. The work has important implications for both immune class regulation and vaccination.

The experiments presented in this thesis demonstrate that vaccination dose is a dominant factor in determining the Th1/Th2 nature of the immune response to BCG. This is a significant observation that makes sense with regard to the functions of the various classes of immunity. The results also demonstrate the property of *coherence* in the immune response to complex organisms (Bretscher, 1994). The immune system appears to have a mechanism for ensuring that the majority of T cells responding to a complex organism are of the same Th phenotype. Such a mechanism is important to ensure that an effective immune response is generated against the infecting organism.

Because TB is a disease which appears to be preventable by a cell-mediated immune response, these results suggest that BCG vaccination for TB may be rendered more effective by administering the vaccine at a low dose, as such vaccination would induce a response that is predominantly of the Th1 type. Furthermore, the administration of low doses of BCG at an early age appears to reduce the effect of exposure to environmental mycobacteria on the immune response, suggesting an approach by which the problem of exposure to environmental mycobacteria could be overcome. Due in part to the results presented here, the Saskatchewan Research Center for the Elimination of Tuberculosis is undertaking a study to examine the efficacy of neonatal low dose BCG immunization in First Nation children.

Immunization with recombinant BCG that express proteins of infectious agents has been a goal of researchers for some years. The method offers some promise as recombinant proteins expressed in BCG generally induce significant immune responses. I have demonstrated that low dose BCG vaccination induces a Th1 response to the expressed foreign protein while higher doses, in this study and in others, induce a mixed Th1/Th2 response. I suggest that low dose immunization with rBCG may be a more effective alternative for vaccinating against organisms that can only be eliminated by cell-mediated immunity, such as HIV and leishmania, than immunzing with the higher doses used in most studies. Moreover, the method may be useful in other special situations where induction of a cell-mediated immune response would be beneficial, such as immunizing against the conserved proteins of influenza virus to provide heterotypic viral immunity.

The opportunity also exists to use rBCG expressing a number of proteins from various pathogens as a multivalent vaccine. This would allow for immunization against numerous possible pathogens with a single vaccine dose. As BCG is considered to be the safest vaccine in use today, the method may provide a safe means to ensure a potent immune response with minimal side effects. Immunization with rBCG containing

numerous foreign proteins may not be necessary initially, as immunization with a low dose of non-recombinant BCG should be sufficient to establish immune deviation. Vaccination with rBCG expressing proteins from various pathogens could be carried out subsequently, as it is expected that the immune response to the recombinant protein would be similar to that induced against BCG proteins due to the property of coherence.

There are many directions that future studies based on the work reported here could take. The observations presented here outline a property of the immune system that could be exploited in a variety of situations. The method offers promise for influenza virus vaccination and future plans of Dr. Qualtiere include expression of portions of the NP protein and/or other conserved influenza proteins in BCG to determine the efficacy of this method in providing immunity to the virus. This model is intriguing because it offers the benefit of an animal model in which to determine protection.

HIV is another virus for which this vaccination procedure has potential. Assessing protection of macaques from infection with simian immunodeficiency virus after immunization with rBCG expressing SIV proteins would be an appropriate model for assessing the potential of the low dose rBCG vaccination method for HIV (Salk et al., 1993). Plasmids that express the SIV gag and env proteins have been acquired from MedImmune.

Expression of the leishmania gp63 surface protein in BCG has been achieved previously (Connell et al., 1993). Immunization of BALB/c mice with rBCGgp63 gave little protection against challenge with *L. major* promastigotes. In this study, a high dose of rBCG was used. I propose that a lower dose of rBCG would be more effective in protecting BALB/c mice, as it is likely to induce a Th1 response to the gp63 protein and hence provide protection to leishmania. Moreover, if low dose BCG vaccination with BCGgp63 is effective in deviating the immune response to gp63 towards Th1, it may even provide a mechanism for treating leishmania infections.

There are applications for low dose rBCG vaccination outside of the field of infectious diseases. Using rBCG expressing allergens to achieve immune deviation of the Th response to these allergens to the Th1 type may allow vaccination against allergy. Exposure to BCG has been shown to divert the immune response to other antigens toward the Th1 pole (Herz et al., 1998; Shirakawa et al., 1997). This is thought to be a consequence of immune regulatory properties of mycobacteria that can induce potent Th1 responses. BCG immunized children were less likely to develop allergy than those who had not been vaccinated (Shirakawa et al., 1997). I believe, as originally suggested by Dr. Bretscher, that vaccination with rBCG containing genes for allergenic proteins would be extremely effective for preventing allergies.

There is increasing evidence that many tumors contain tumor-specific antigens (Boon, 1992; Gaugler et al., 1994). Immunization with low numbers of rBCG expressing tumor-specific antigens may provide a means for vaccination against tumors.

In conclusion, I believe that the experiments presented here will have extensive application and should provide many possibilities for further productive research.

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